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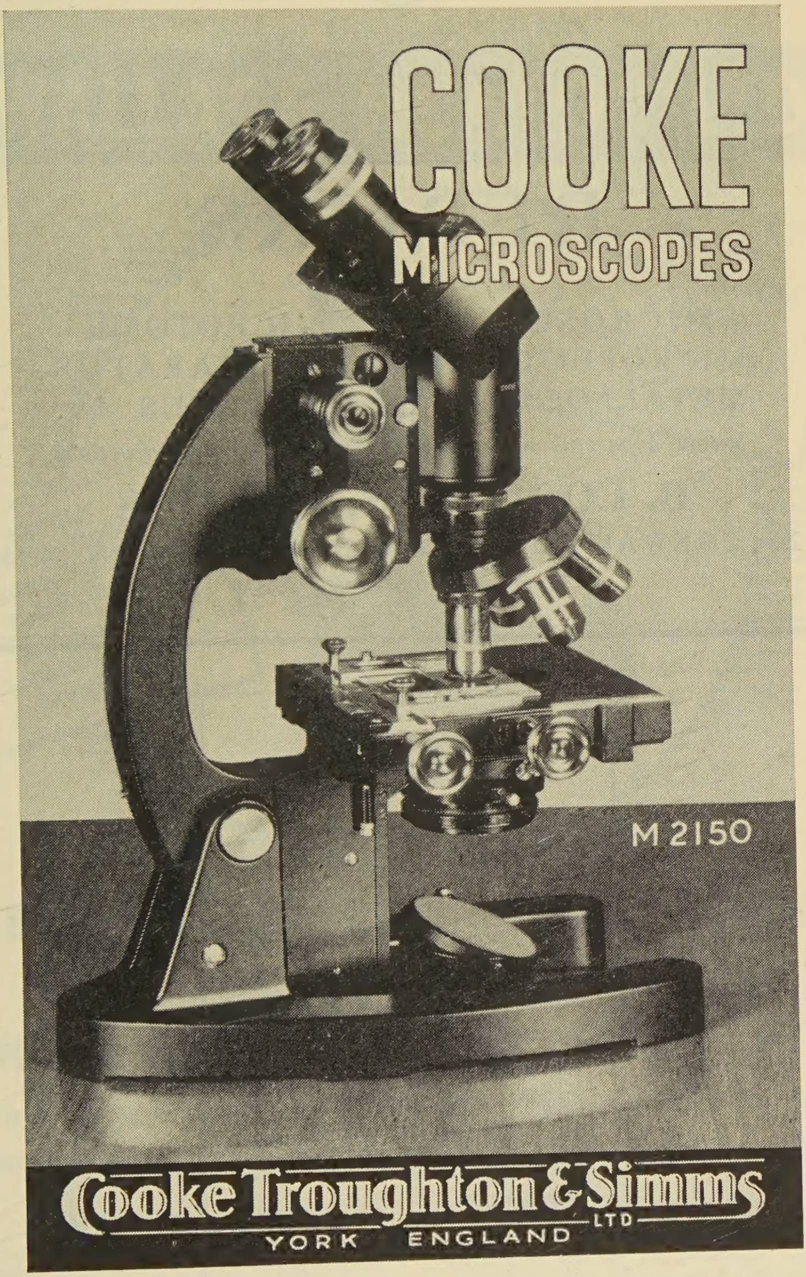
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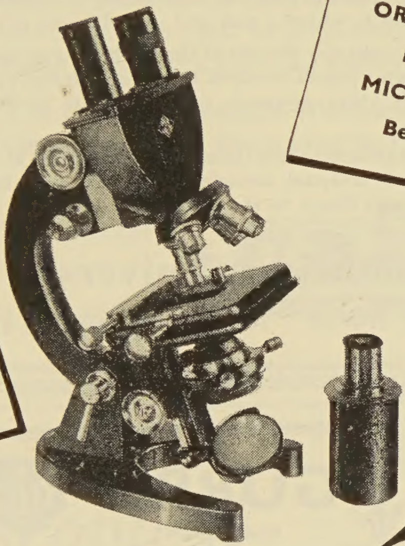

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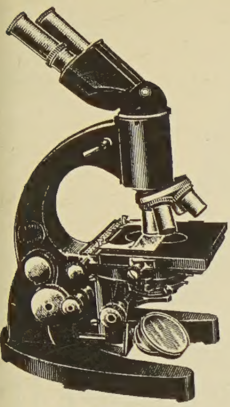

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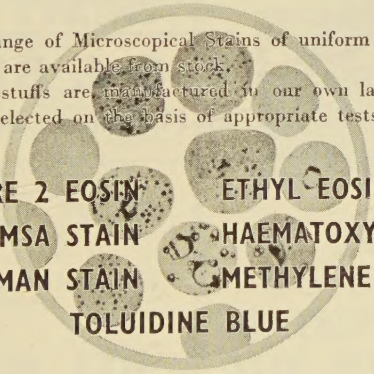

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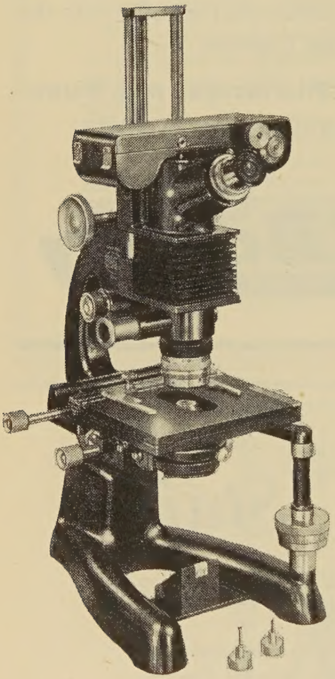

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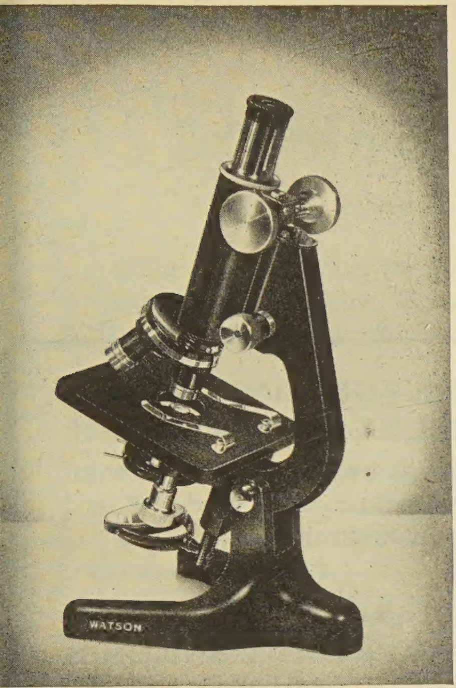

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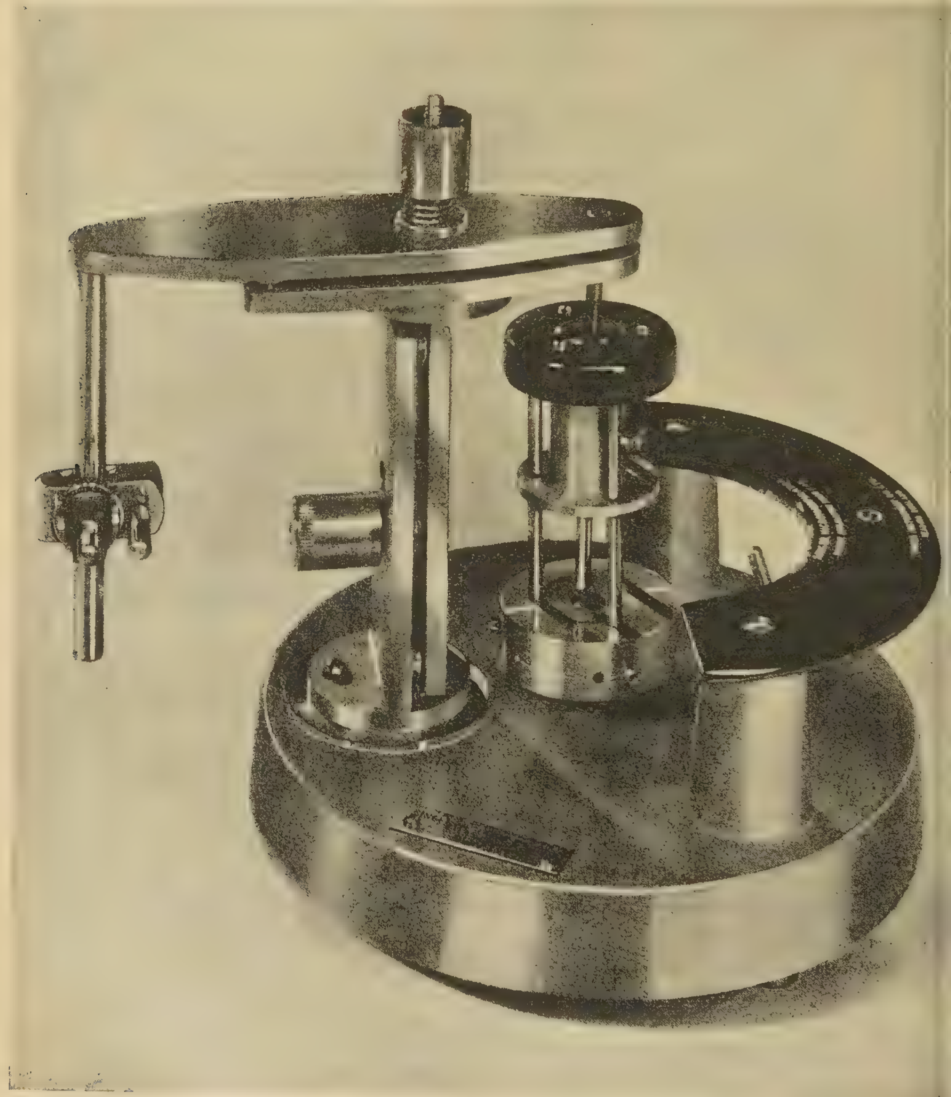

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Experiments on Vital Staining with Methylene Blue

By JOHN E. HARRIS AND A. PETERS

(*From the Department of Zoology, University of Bristol*)

SUMMARY

A new method is described for vital staining of nervous tissue with methylene blue; it involves impregnation of whole embryos or tissue-fragments with the rongalit-reduced dye under anaerobic conditions at pH 5, followed by development of the stain in an oxygenated neutral saline solution. Determinations of the solubility of leucomethylene blue and experiments on vital staining with it and with the oxidized dye at different pH values are consistent with the hypothesis that the dye penetrates cell membranes and tissues mainly as the leucobase, but may pass along nerve (and muscle) fibres in the oxidized form. Penetration of the leucomethylene blue below pH 5 is hindered by its ionization; above pH 5 the penetration falls off because of reduced permeability of the cell membranes.

SOME four years ago, with the help of Miss L. Morris, one of the present authors developed a new method of vital staining of the nervous system of the embryo dogfish, *Scyliorhinus canicula*, using a modification of the old-established rongalit methylene blue technique. The success of this method led to an experimental study of some of the factors involved, and the present account deals mainly with these studies. The work is concerned almost entirely with the penetration of the stain into the tissues; the part, if any, played by the specific chemical combination of nerve-cell constituents with the dyestuff in its final site is not considered.

Since the method used originally on the dogfish has not previously been published, and has been very little modified as a result of the later work, it will be convenient to outline it first.

A TWO-STAGE METHOD FOR VITAL STAINING WITH RONGALIT METHYLENE BLUE

The stock solution of rongalit-reduced methylene blue was prepared according to the method of J. E. Smith (1947).

A dogfish egg-case was cut open and the yolk together with the embryo was carefully transferred to a dish of dogfish saline (Bialaszewicz, 1933). The embryo was dissected off with as little damage as possible, any surplus yolk being removed by a jet of water from a fine pipette; it was then transferred to a thick-walled glass test-tube containing 10 ml. of the saline. A well-fitting rubber stopper, pierced with a single hole, sealed the tube mouth and served to connect the tube to a gas line including a simple mercury manometer, a rotary vacuum pump, and a gas-cylinder of nitrogen (oxygen-free).

[Quarterly Journal of Microscopical Science, Vol. 94, part 2, pp. 113-124, June 1953].

The air in the tube was evacuated; tapping the walls of the tube helped t liberate dissolved air, and the pressure was reduced until the solution jus began to boil at room temperature (15–20° C.) As soon as this occurred th vacuum line was shut off and nitrogen admitted until atmospheric pressur was re-established. The stopper was removed, and 0·1–0·5 ml. of rongali methylene blue stock solution was added, the tube being closed again as soon as possible. The evacuation and admission of nitrogen was repeated to remov the last trace of oxygen and the system was then left for about an hour for impregnation of the embryo with the dye (or rather with its leuco-derivative to be completed.

Within a minute of the deoxygenation the embryo ceased to show any of it typical rhythmical movements. At intervals during the hour the contents o the tube could be agitated by rotating it between the palms of the hands; th actual time used for this stage, and the amount of rongalit methylene blu stock added, varied with the age of the embryo.

After this phase was completed the tube was unsealed and the contents poured out into a Petri dish. The embryo was taken up in a pipette, and transferred to a dish of fresh well-aerated saline and kept under examination with the high power of a binocular dissecting microscope; it was illuminated (by reflected light) only at intervals. Within a few seconds it became uniformly blue; after a minute or two normal body movements started again. At first there was no visible differentiation of the nerves, but in a time which varied from 10 minutes to half an hour or more, the muscles and other non-nervous elements became paler and more transparent while the nerve cells and fibres took on the typical intense dark blue stain.

The dye used was obtained from Grübler, Gurr, British Drug Houses or Imperial Chemical Industries, and was in each case the zinc-free chloride for vital staining. All brands were successfully used, the only difference being in the extent to which the nervous system was differentiated from other tissue elements, which were sometimes stained to a greater or lesser extent. A rongalit methylene blue (RMB) stock which was pale pink in colour invariably gave better differentiation than one which was yellow, though the nerves were always well stained. We believe that staining of non-nervous elements in these specimens is brought about by other thionine derivatives, probably present as impurities. Heller, Starr, and Davenport (1949) have indicated this and the present writers can confirm that in the case of the dye 'new methylene blue', the use of a rongalit-reduced solution leads to a very well developed staining of other tissue elements without any staining of nerve fibres. It appears that tetramethyl thionine (methylene blue in the strict sense) is highly specific in its nerve staining; the trimethyl, dimethyl and monomethyl derivatives and thionine itself (Lauth's violet) show a decreasing ability to stain nerve cells and fibres together with an increasing affinity for other tissues. Since the lower methyl homologues are present to some extent in all commercial methylene blues (from which in fact they are more or less impossible to remove), it is reasonable to suppose that our Grübler dye sample, which produced a typica pink RMB stock, and gave the best *differentiation* of the nervous system, contained the smallest amount of these impurities. The difference between the four samples was, however, small. While rongalit white supplied by Gurr was used in all the later studies, other batches of different origin and of unknown age were used with equal success in the early work.

Not only was the source of dye and rongalit relatively unimportant, but the ageing of the RMB stock was also immaterial. Kept in a well-stoppered or wax-sealed tube it has been used at all times after preparation, from immediately after it has been filtered up to 2 months later. It is generally stated that the solution should be used only after 24–36 hours and up to 8–10 days (Pantin, 1946). The objection to its immediate use, we believe, is the presence of an excess of hydrogen sulphide in the freshly prepared stock-solution; this penetrates organisms very readily (Osterhout, 1925) and is highly toxic. The evacuation of the staining solution removes the hydrogen sulphide as effectively as it does the oxygen; embryos will remain alive and healthy in it for long periods, whereas they are rapidly killed by an RMB solution which has been used in the normal manner, too soon after preparation of the stock.

The poor results obtained from a stock solution which has been aged too long are partly due to reoxidation of the leucomethylene blue, the solution taking up atmospheric oxygen and turning blue in the process. This does not occur if the stock bottle has a very small free air space and is tightly sealed.

One of the most striking features of the two-stage method of vital staining described above was the almost simultaneous development of the stain in all the nerve cells and their processes, both central and peripheral. It is clear that during the impregnation stage a large and fairly uniform concentration of dye in the reduced condition accumulated throughout the organism. As oxidation proceeded, the dye appeared to migrate into its final site; the muscles and other non-nervous tissues lost their early blue colour while the nerve cells and fibres became more and more intensely stained. So far as we can see, nerve staining was complete in all the functioning neurons and their processes. At what stage in its life a neuroblast becomes capable of concentrating this enormous amount of dye we do not exactly know, but some yet-unpublished work suggests that it is less than 48 hours before it begins to conduct impulses.

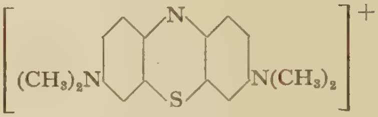
AN EXPERIMENTAL STUDY OF THE IMPREGNATION PROCESS

1. *The equilibrium of reduced methylene blue in the staining solution.*

It was fortunate in many ways that the method had been tried first on dogfish embryos, for, as will become apparent later, the conditions provided by the experiment were accidentally almost ideal for its success. Similar tests with embryos of the lamprey and trout were less successful until it was realized that the lamprey and trout Ringer solutions used were buffered by the addition of phosphate. Owing to the hydrochloric acid added in its preparation, RMB stock solution is acid and the staining solution made with dogfish saline was at about pH 5; that made with the other buffered Ringer solution was much nearer to pH 7. Clearly an investigation of the effect of pH on th impregnation was required, and also a study of the dissociation of the leucomethylene blue under the conditions of impregnation.

According to the work of Clark, Cohen, and Gibbs (1925) the essentia features of the oxidation-reduction equilibrium of methylene blue may b briefly summarized as follows: methylene blue chloride (the form in which it is used for vital staining) exists in solution in the oxidized form as a completely dissociated salt at all pH values of biological significance.

The structural formula of the cation may be written:



Reduction of this radicle gives leucomethylene blue by producing a negative charge on the bridging N-atom, which fixes a hydrogen ion in consequence. This gives an uncharged molecule which we may write as HB, where B is the radicle shown in the structural formula above (neglecting changes in electronic configuration brought about by the reduction). The unionized leucomethylene blue HB is colourless and forms the principal component present in neutral or alkaline reducing solutions. In acid solutions, however the leucobase adds on first one and then a second hydrogen ion to the dimethylamino-groups, giving radicles which can be schematically represented by the formulae H_2B^+ and H_3B^{++}.

The unionized leucobase HB is poorly soluble in water and in physiological saline solutions. Clark, Cohen, and Gibbs (1925) give approximate figures for its solubility at pH 2·9 and 8·6; they also made careful and detailed potentiometric titrations to establish the dissociation constants of the leucobase. These dissociation constants we have used in the calculations below, but it has been necessary to obtain accurate values of the solubility of the leucobase over a wider range of acidities than in this early work.

This solubility has been estimated by three different methods: (*a*) by a direct colorimetric estimation of methylene blue after oxidation of a saturated leucobase solution, (*b*) by weighing the methylene blue precipitated when such a solution was treated with an excess of potassium dichromate, and (*c*) by a direct titration of the leucobase solution against 2, 6-dibromophenol indophenol in an atmosphere of nitrogen. Each value for the solubility at any given pH was checked by two of the three methods, method (*b*) being appropriate in acid solutions where the solubility is high, and method (*c*) at alkaline pH-values where the redox indicator is most reliable. The results are summarized in graphic form in fig. 1, which also shows the calculated concentrations of HB, H_2B^+, and H_3B^{++} present in an actual staining solution in which the embryos were immersed (assumed to be 0·5 ml. of RMB stock in 10 ml. of saline at 18° C).

It will be seen that from pH 9 down to pH 6 the concentration of leucobase in the staining solution is substantially constant; the solution is saturated, free precipitated leucobase being present, as the staining experiments show. Below pH 6 the amount of ionized H_2B^+ becomes appreciable; that of H_3B^{++} rises at and below pH 5. On the acid side of pH 5 the concentration of undissociated leucobase, HB, falls off very rapidly; the total soluble leucobase

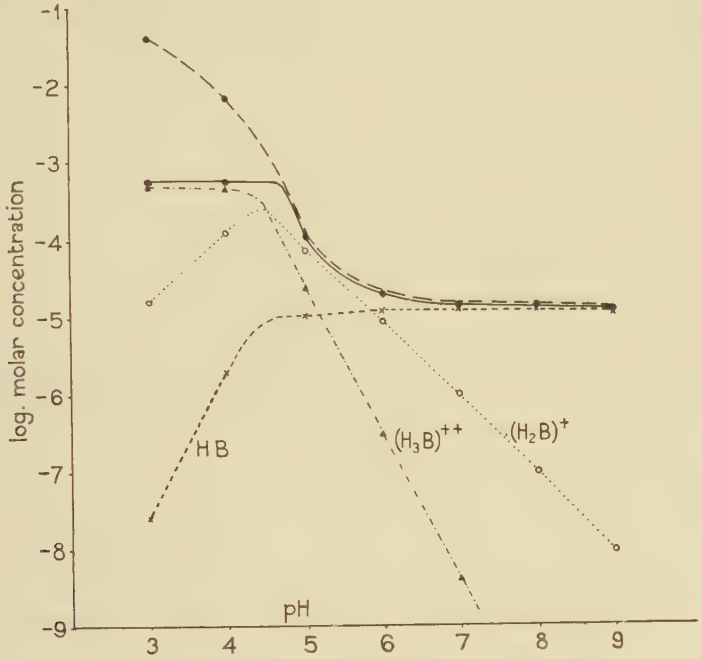


FIG. 1. The equilibrium of reduced methylene blue in aqueous solution.

•— — — —• experimentally determined solubility of leucomethylene blue.

•————• total dissolved leucomethylene blue in a standard rongalit staining solution containing 6×10^{-4} *M* methylene blue. Above pH 4·5 precipitated leucobase is present.

HB
x- - - - - - - -x
$(H_2B)^+$
o·····o
$(H_3B)^{++}$
▲—·—·—·—▲
concentrations of the three separate components present in the standard solution.

$(HB + H_2B^+ + H_3B^{++})$ increases until pH 4 is reached, below which value the whole of the reduced dye is present in true solution, principally as the divalent cation H_3B^{++}.

2. *The effect of hydrogen ion concentration on staining.*

The first experiments were simply an extension of the new method to investigate the effect of pH on the uptake of dye under reducing conditions. Owing to the difficulty and expense of obtaining a large number of similar dogfish embryos, the work was done on pieces of earthworm body-wall, each including a portion of the ventral nerve cord. Each experiment involved seven similar pieces of tissue taken from a single worm. The pieces were placed singly in 10 ml. of earthworm saline (Pantin, 1946), made up without phosphate; the pH of the seven solutions was adjusted to 3, 4, 5 . . . 9 by the addition of NaOH and HCl or by adding 2 ml. of standard buffer (Clark and Lubs, McIlvaine, or Kolthoff buffers were used for this purpose; see Clark, 1923). Whenever buffer solutions were not used, the pH was rechecked electrometrically at the end of each experiment; tests showed that there was no appreciable change during the process of uptake of the dye.

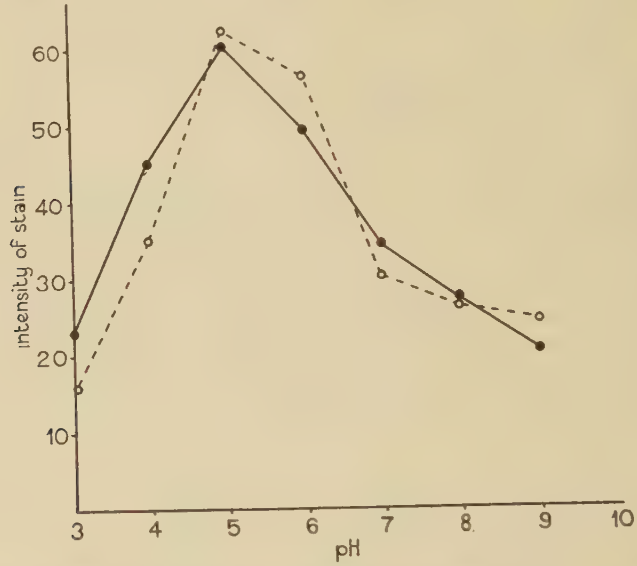


FIG. 2. Staining of earthworm tissues with rongalit methylene blue under anaerobic conditions. •——• nerve cells and fibres. o------o muscles. (For method of calculating ordinate, see text.)

At the end of a fixed period, identical for all seven specimens, but varying from 30 minutes to 5 hours in different experiments, each preparation was dropped into a solution of sodium perchlorate, which served to oxidize and fix it; it was then mounted in a gum acacia glycerine medium of refractive index 1·51–1·52 for examination.

Since little or no time was allowed for differentiation to occur, muscle and nerve were both stained; the seven preparations in each experiment were arranged in descending order of intensity of staining. To the best preparation was given a 'mark' of 7, the remainder being rated 6, 5 . . . down to 1.

The results of twenty-three experiments on 150 specimens are summarized in graphical form in fig. 2, which relates the 'total marks' in all experiment to the pH of impregnation, irrespective of the method of buffering and tim of impregnation. It is clear that the maximum intensity of stain in both nerv and muscle occurred at pH 5. In fact, pieces stained at pH 3 and 9 were almos colourless; neither the optimum pH nor the intensity of stain showed any systematic variation with the method of buffering employed.

In the above experiments we were not concerned with the differentiation of nervous tissue from muscle; the similarity of the curve of staining in the two tissues suggests that the main factor involved is the rate of penetration of the dye through the organism, which is most rapid at pH 5. We have shown that below this value the concentration of undissociated leucobase falls off; below pH 5, though the total solubility of leucomethylene blue increases rapidly, it is largely present as the ions H_2B^+ or H_3B^{++}.

When a whole organism is immersed in an oxygenated methylene blue saline solution, staining takes place in a very different manner. Penetration of the dye is very slow, except at points where the surface is damaged; from such regions a selective staining of nerve fibres takes place more or less rapidly, the non-nervous tissues being almost uncoloured. Along the peripheral nerve fibres the stain develops until it reaches the neurons in the central nervous system, through which it spreads in a similar selective manner, apparently through 'functional' contacts of each neuron with its physiologically linked neighbouring neurons and their fibres. The method is in fact a useful one for exploring the innervation of a peripheral structure. Since penetration of the dye in this way takes place by a different path, it seemed useful to investigate the effect of pH on this type of staining on the same material.

Pieces of earthworm body wall immersed in oxygenated saline containing methylene blue stain progressively from the cut edges; only the damaged muscles are stained appreciably, but nerve-staining extends centrally as described above. Fig. 3, obtained from a second series of twenty experiments with 140 specimens stained in this way and assessed on regions within the restricted area of satisfactory staining, shows that the best staining of the nerves occurred in alkaline solutions with an additional peak at pH 7; below pH 7 the intensity fell off until the preparation was almost colourless at pH 3. For the damaged muscles, the intensity of staining fell off progressively from pH 9 down to pH 3; once again there was no systematic difference that could be ascribed to different buffer solutions.

The very different pH optima shown by these experiments can be reconciled with that shown under reducing conditions if we assume that the final staining in the last experiment is a resultant of two distinct processes: (*a*) the penetration of the dyestuff along the nerve or muscle fibre, which does not involve the crossing of a cell boundary, and (*b*) the loss of dye from the stained nerve or muscle through the cell membrane into the surrounding unstained tissues. The latter process does demand the passage of the dye through a cell membrane, which we assume takes place almost entirely in the form of unionized leucomethylene blue. In the living cell there must always be an equilibrium between the oxidized and reduced form of the dyestuff; the amount of staining will be less if reduced dye molecules are constantly leaching away. This leaching out is most likely to occur at acid pH values provided that these are not so acid as to ionize the leucomethylene blue.

The pH of the neighbouring tissues is never likely to fall as low as that of an acid external medium; poor staining with oxidized methylene blue in acid media may be explained by loss of leucomethylene blue from stained cells if the permeability of the tissues to the leucobase increases on the acid side of neutrality. This result appears to contradict the earlier hypothesis, but a reference to the first experiment illustrated in fig. 2 supports this conclusion. The solubility of the leucobase is only slightly less at pH 9 than it is at pH 5; yet we see from fig. 2 that the staining (from RMB solution) falls off very greatly, not only below pH 5 where the leucobase ionizes, but also above pH 5 where it does not.

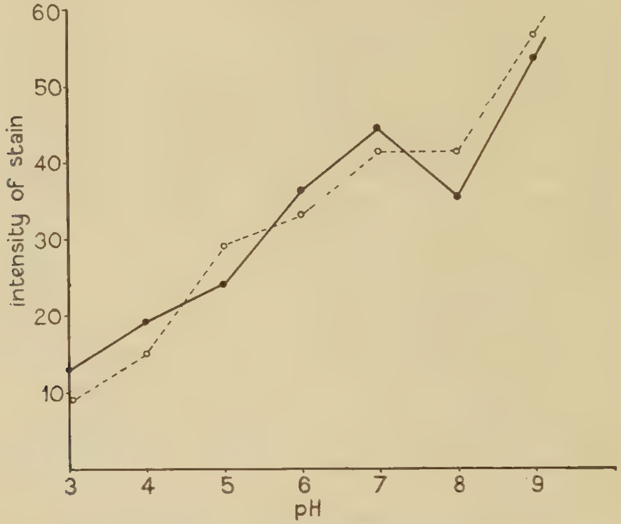


FIG. 3. Staining of earthworm tissues with methylene blue under fully oxygenated conditions; •———• nerve cells and fibres. o-------o muscles. (For method of calculating ordinate, see explanation to fig. 2. in text.)

The reduced permeability of the tissues to leucomethylene blue as the pH rises above 5 required additional confirmation; a third series of experiments was designed to investigate this point. Pieces of earthworm were impregnated in RMB solution under anaerobic conditions at pH 5. At the end of a definite period of impregnation they were transferred to a series of tubes containing deoxygenated saline (without rongalit or dye) at pH values from 3 to 9. After a further period in these buffered saline solutions the specimens were removed and fixed in sodium perchlorate as before; one specimen, fixed immediately after it was taken out of the RMB solution at pH 5, served as a control.

The results of these experiments showed a rapid loss of leucomethylene blue into the saline solutions at all the lower pH values. At pH 9 and 8 there was almost no loss of stain, compared with the control, but at all values below pH 8 the loss became progressively greater. When the 'impregnation time' and 'leaching-out time' were equal, tissues leached at pH 3 and 4 became completely colourless; if the impregnation time was rather less than the leaching-out time the preparation at pH 5 became colourless, and so on.

When the same experiment was tried with fully impregnated stained tissue leaching into *oxygenated* buffered saline solutions, only a very small loss of dye occurred even below pH 5.

It is important to realize that these tissues had originally been treated with saturated reduced methylene blue at pH 5; they had therefore accumulated a large amount of the dye throughout. In the earlier experiment reported (fig. 3), poor staining had been noted in acid oxygenated methylene blue at pH 5. Here, however, penetration of oxygenated methylene blue was limited to the 'exposed' nerve processes and cells; internal loss of leucobase could therefore occur to the very large volume of unstained neighbouring cells.

The rapid loss of reduced dye in solutions of low pH suggested the possibility of obtaining a very rough quantitative estimate of the amount of dye taken up. Pieces of earthworm were impregnated in deoxygenated RMB saline at pH 5; after 2, 3, and 5 hours they were transferred to fresh dye-free deoxygenated saline at pH 3. The solution obtained was allowed to oxidize and its dye content was estimated colorimetrically; from the wet weight of the pieces of worm used, an approximate estimate of the dye concentration in the tissue could be obtained. In seven experiments this was found to vary from $1{\cdot}5 \times 10^{-3}\ M$ to $2{\cdot}1 \times 10^{-3}\ M$, showing no correlation with the time of impregnation; evidently equilibrium was attained in less than 2 hours. The average dye concentration ($1{\cdot}9 \times 10^{-3}\ M$) in the wet tissue was about 17 times that in the original saline at pH 5 ($1{\cdot}1 \times 10^{-4}\ M$), suggesting that even for leucomethylene blue there is a genuine accumulation of dye in the tissue. Clearly in the fully differentiated stained nerve fibre the concentration of oxidized dye must be enormously higher than this.

Experiments on earthworm fragments stained with RMB saline solutions in air (i.e. as in the normal rongalit staining process recommended by Pantin, 1946) gave inconclusive and highly variable results between pH 5 and pH 9; below pH 5 there was little or no staining. This inconclusiveness is not surprising; it is very difficult to achieve constant and reproducible conditions in dealing with a reducing solution in air, as anyone who has used the ordinary rongalit method on large specimens will appreciate. In addition a new factor enters into these experiments. The rate of oxidation of leucomethylene blue is a function of pH; it is relatively stable under acid conditions but the rate of oxidation, according to Clark, Cohen, and Gibbs (1925), is proportional to the fifth power of the OH-ion concentration. In the early stages of rongalit staining in air, the reduced dye will pass into the tissues as the leucobase, but at physiological pH values in the tissues it will be rapidly oxidized and will travel in the oxidized form along nerve fibres without crossing a cell membrane. Selective staining of nerve elements will therefore occur from the surface inwards, a result typical of the normal rongalit staining method.

It is clear that in the normal process of rongalit methylene blue staining, one of the functions of the acid in the staining solution is to increase the permeability of the organism to the (reduced) dyestuff; another function may well be to depress the rate of oxidation of the leucobase in the saline medium.

Discussion and Conclusions

The experiments described have suggested the following general conclusions.

In pieces of excised tissue, and in whole organisms where surface damage has produced similar conditions, oxidized methylene blue stains nerve cells and fibres (and muscle cells, though less strongly), with increasing speed and intensity as the pH of the staining solution is raised. In such experiments the dye penetrates along intracellular pathways in the nervous tissue, not crossing cell boundaries except at functional (synaptic ?) contacts, which may well represent regions of alteration in properties of the membrane. A simple, though not necessarily correct, explanation of these facts could be made on the assumption that the free dye cation of the completely ionized methylene blue salt combined with negatively charged proteins (Singer, 1952).

Reduced methylene blue penetrates and stains such tissues, as well as undamaged whole organisms, much more readily, crossing cell-membrane barriers in the process. It is suggested that this penetration occurs in the form of uncharged molecules of leucomethylene blue. Below pH 5 the rate of penetration falls off very rapidly; this result agrees with a corresponding diminution in the concentration of uncharged leucomethylene blue, which ionizes at more acid values to give successively a singly and a doubly charged cation.

Unlike the oxidized dyestuff, reduced methylene blue is most rapidly taken up at this critical value of pH 5. Above this value penetration of leucomethylene blue falls off with increasing alkalinity. This is true even though the external concentration of the slightly soluble leucomethylene blue remains roughly constant above pH 5; under the conditions employed the solution was saturated. Between pH 6 and pH 9 the solubility of leucomethylene blue was estimated to vary only from $1{\cdot}17 \times 10^{-5}\ M$ to $1{\cdot}45 \times 10^{-5}\ M$, a variation which was probably within the experimental error of the determination.

At pH values above 5, the rate of loss of reduced dye from previously impregnated fibres parallels the rate of uptake by unimpregnated ones. We are therefore not dealing with an affinity of the nerve fibre for the leucobase; the changes in rate of uptake and loss must reflect an effect of pH on the permeability properties of the cell membranes to the uncharged leucobase molecules.

There is little evidence that the positive ion of oxidized methylene blue can penetrate or leave undamaged living cells. Since, however, it must be in equilibrium with its reductant, the final colour of any stained preparation will depend not only on the affinity of the cell constituents for the oxidized dye, but also on the concentration of reductant which is in equilibrium with it; this will control movement of the reductant into and out of the cells in question. So long as the cells are intact, all these considerations will favour retention of stain at high pH values. Under such conditions the ionization of the amino-groups of the proteins will be depressed, and their negative charge increased; also, the permeability of the cells to the small amount of leucomethylene blue in equilibrium within the stained cells will be reduced, limiting the escape of dye into other neighbouring cells.

The successful staining of intact organisms with methylene blue under aerobic conditions will be determined by similar complex considerations. Even when the bulk of the staining solution is fully oxygenated there will be, close to the surface of the organism, an appreciable concentration of reduced leucomethylene blue, which can penetrate the surface membranes. Once in the cells it will come into equilibrium with the oxidized cation which we assume combines with the cell proteins. Within the organism as a whole, oxidized methylene blue (i.e. stain) will accumulate (*a*) in regions in which the affinity for the cation is greatest, as with those proteins farthest on the alkaline side of their isoelectric point, and (*b*) in regions where the steady state equilibrium of the oxidant-reductant system is shifted most towards the former. Both of these characteristics may occur in living nerve cells; which of the two is the more important factor in the selective vital staining of nerve cells with methylene blue requires further investigation.

The conclusions outlined above are based largely on the experiments carried out in the present study, but they are in line with much of the earlier work with methylene blue as a vital stain. The contention of Irwin (1926) that the dye penetrates *Valonia* only in the form of oxidized lower methyl homologues appears unnecessary, but this view had already been criticized by M. M. Brooks (1927). It is pointed out by Brooks and Brooks (1941) that the dye is largely reduced in the cell protoplasm of *Valonia*, accumulating in the blue oxidized form in the central vacuole, so that our suggested mechanism of penetration may well apply to this alga. The fact that methylene blue penetrates *Valonia* faster from solutions at pH 8 than at more acid values (Brooks, 1926, 1927, 1929) agrees with our own data for the oxidized dye; in commenting on this result it is interesting to find that Brooks and Brooks (1941) say 'the difference in rate may be due to effects of pH on the plasma membrane rather than on the dye', but they do not appear to envisage penetration of the dye as the undissociated leucobase. There appears to be very little work on the effect of pH on permeability to such non-electrolytes. Davson (1939) has shown that haemolysis of rabbit red cells by glycerol is most rapid at pH 5, but Danielli (1943) emphasizes the abnormality of the conditions in these experiments, carried out in almost electrolyte-free solutions.

The most recent study of the effect of the medium on vital staining with methylene blue was reported by Heller, Thomas, and Davenport (1947), who worked on similar lines to those studied by Schabadasch (1936), but investigated staining by immersion instead of by injecting the dye into the blood circulation. Heller and others found the optimum pH to be about 5·6 in the oxygenated staining solution. This is nearer to our optimum for the reduced dyestuff but it may well be that even in Heller's experiments the actual penetration was in the form of leucomethylene blue. The favourable effects of acetate, lactate, pyruvate, &c., reported by Schabadasch and Heller, reflect the probable importance of metabolic factors in the oxidation-reduction equilibrium, which we believe may be more particularly significant in the subsequent differentiation of the tissues than for the penetration of the stain.

One point in the present series of experiments has been only lightly touched upon. In wholly reducing anaerobic conditions leucomethylene blue accumulates in the tissues at a much higher concentration than in the surrounding medium. Stoichiometric combination of the dye cation with, or its adsorption on, an amphoteric tissue constituent as in the experiments of Pischinger (1927) and Singer (1952) cannot explain concentration of the uncharged leucomethylene blue; one is tempted to think of the process in terms of lipoid solubility. Though methylene blue is quoted by Brooks and Brooks (1941) as a lipoid insoluble dyestuff, it seems probable that they are referring to the oxidized form, which as a hydrated cation would be expected to have a low oil/water partition coefficient. The reverse is in fact true for the leucobase. A rough experiment with chloroform/water equilibration under anaerobic conditions showed a concentration ratio of the order of 25:1 in favour of the chloroform fraction.

We wish to acknowledge the help given by Miss L. Morris in the early stages of development of the staining technique. The later part was carried out during the tenure by one author (A. P.) of a graduate scholarship in the University of Bristol.

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Cell Shrinkage caused by Fixatives and Paraffin-wax Embedding in Ordinary Cytological Preparations

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SUMMARY

A series of measurements was made of the diameters of the cells and nuclei of the primary spermatocytes of the snail *Helix aspersa* in preparations made by embedding in paraffin wax after fixation in a number of different cytological fixatives. These were compared with similar measurements made on the living spermatocytes mounted in suitable saline solutions, and the shrinkage that the cells underwent as a result of fixation and embedding in each case was calculated from the medians of the size-distributions recorded.

The results showed that the amount of shrinkage varies to some extent according to the fixative employed—picric acid gave the greatest linear cell-shrinkage (about 40 per cent.) and Sanfelice's fluid by far the least (about 13 per cent.); but with this one exception the differences in final cell-size were not very great, and represent a linear shrinkage to about 67 per cent. and a volume shrinkage to about 30 per cent. of the original dimensions of both cell and nucleus.

INTRODUCTION

THE object of this work was a strictly practical one, namely, to measure to what extent the cells seen in ordinary fixed and stained microscopic preparations have shrunk from their size when alive, and how this varied according to the fixative used.

The results obtained may bear to some extent on current theory about fixation, but they will not do so directly, since, although in some cases the fixative is probably responsible for most of the shrinkage in a preparation, any embedding process is an additional modifying factor in reducing the final cell-size still farther, and paraffin-wax embedding after alcohol and xylene or toluene—by far the commonest method, and the one used here—is certainly one of the most drastic processes in this respect. As it happens, measurements of changes in cell-size under the effect of fixatives alone have been made at the same time by Crawford and Barer, and their results, when fully published, should cover this aspect very adequately.

In fact it is rather surprising to find how little work has been done hitherto on size-changes in actual cells. It is true that very similar studies have been made by Berg (1908) and Tarkhan (1931) on pieces of tissue, and by Patten and Philpott (1922) on whole embryos, measured before and after fixing; but not very much can be inferred from these results, because many of the extracellular substances, which form a considerable proportion of these larger

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bodies, may react with fixatives in an entirely different manner from the protoplasm of the cells. Sometimes they are practically inert. In addition, some organs may have large intercellular spaces which usually tend to collapse after paraffin embedding, and cavities are, of course, always present in whole organisms.

In fact, the only really comparable work appears to have been done by Kaiserling and Germer, who in 1893 measured egg cells from cow ovaries, but this was partly invalidated by their unfortunate use of a hypotonic salt solution in the course of their technique—nor was their fixed material subsequently embedded. Thus it was with an eye to filling a gap in elementary data that this present work was attempted.

MATERIAL

It was decided to use the primary spermatocytes of the snail *Helix aspersa*, the gonad of which was invariably found to contain primary spermatocytes at all stages of development in spite of the fact that the work was done in the middle of the winter. The ovotestis was always very small at this time and it was seldom possible to make more than one or two preparations from a single organ; this, however, did serve to establish that there were no very great differences in cell-size between different individuals. In addition the cells themselves have the following advantages:

(1) They are easily recognizable in all preparations—the only other round cells present being Sertoli cells, which are heavily granular and usually larger, and the quite distinctive secondary spermatocytes and young spermatids, which are relatively uncommon in winter.

(2) They have no very specialized organelles and the ratio of nucleus to cytoplasm is fairly normal. They resemble the 'typical animal cell' (in so far as such a concept may be valuable), and, while not very much larger than the average, they are at the same time large enough for dimensions to be measured accurately at least with no larger error than 7 per cent. of the whole, with the technique employed.

(3) The cells are spherical or nearly spherical and in the majority of cases show no great distortion after fixing and embedding, which means not only that only one diametrical measurement is necessary, but that estimations of volume and surface area based on nuclear- and cell-diameters also have validity within the limits of accuracy of the work.

METHOD

During dissection the animal was not placed in any saline solution. The ovotestis was removed rapidly and a portion of it placed for at least 6 hours in the fixative and then passed through alcohol and toluene and embedded in paraffin wax (1 part of melting-point 54° C. to 3 parts 52°). 8-μ sections were cut and the preparations were subsequently stained in iron haematoxylin and mounted in Canada balsam, the whole process being standardized throughout. (The 'post-chroming' and iodine treatments often employed after the use of reagents containing chromium and mercuric chloride respectively were omitted, since it did not seem likely that they, or any other treatment subsequent to embedding, would affect cell-size.) Cell-diameters were then measured with an eyepiece micrometer and a 2-mm. oil-immersion objective. The diameters of fifty cells and their nuclei were thus measured in each preparation—often all in a single section—and their size-distribution compiled in histogram form with eyepiece divisions forming the units of the abscissa. The microscope was actually set up so that one eyepiece-division equalled $1 \cdot 25\,\mu$; but in estimating shrinkage in percentages, absolute units are, of course, unnecessary.

These results were compared with the size of living cells similarly observed in teased preparations.

The work was then repeated, generally with fresh reagents and snails from a different locality. As the results obtained did not differ markedly from the previous ones, the two were combined and presented as the sum of 100 cell-measurements. Just how far this step was in fact justified will be discussed below.

PROBLEMS OF THE TECHNIQUE

Various difficulties, however, cropped up as the work proceeded which might have cast doubt on its final validity, and these will have now to be discussed in fuller detail.

Of these, perhaps the most important lay in the fact that in one respect the fixed and living material were not exactly comparable, namely, in the relative numbers of the smaller cells. The ovotestis is a partitioned structure with the larger, more fully developed spermatocytes floating freely in the cavities, while the smaller cells usually lie close to and often adhere to the walls; and, naturally, in teased preparations these tend to get left behind in the debris and fewer are included. This, coupled with the fact that the smaller cells are in any case rather numerous, made it very desirable to devise some criterion, other than size alone, by which the juveniles could be rejected and the study confined to the larger and more mature groups. Fortunately this was possible owing to the fact that during growth the size of the nucleus does not increase proportionately to that of the whole cell: the larger cells have relatively much more cytoplasm, while in the smaller size-groups the diameter of the nucleus approaches very closely the diameter of the whole cell, and often the difference between the two is less than one eyepiece division ($1 \cdot 25\,\mu$). Thus, by measuring only those cells with a measurably different diameter of cell and nucleus, one's object is largely achieved. I am grateful to Dr. D. I. Finney of the Bureau for the Design and Analysis of Scientific Experiments (Oxford) for his advice as to the justifiability of this step. It is, however, open to the objection that the assumption is made that the nucleus and cytoplasm shrink equally as a result of fixation and embedding, which is not quite true. (In practice, as will be seen, the nucleus usually shrinks slightly less than the cytoplasm.)

I am indebted to Dr. C. F. A. Pantin for pointing out a second important

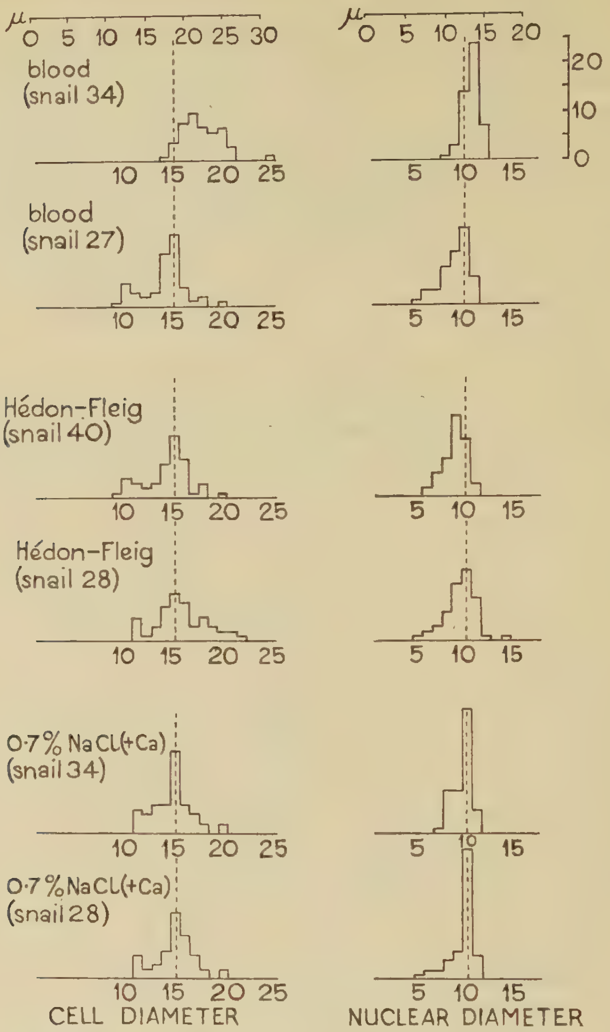

FIG. 1. Frequency polygons showing the size-distribution of living *Helix* primary spermatocytes in snail's blood and various saline media. Fifty cell measurements and fifty nuclear measurements from each preparation.

In this and in all the other figures, the vertical scale (at top right) shows the number of measurements; it is applicable to all the polygons in the figure. The numbers under each polygon constitute a scale of eyepiece-divisions (1 division = 1·25 μ); corresponding scales in μ are drawn at the top of the figure.

difference affecting the comparison of size measurements in the fixed and living preparations, and this is that, owing to the aqueous (saline) mounting medium of the latter being of lower refractive index than Canada balsam, the objective has a shorter focal length, and living cells measured in the manner described are bound to appear slightly larger than in balsam; and an experiment had to be performed to investigate the importance of this effect. The engraved scale of a stage-micrometer was measured with an eyepiece micrometer, first in Canada balsam and then under exactly the same conditions with the balsam replaced by a layer of saline of approximately the same thickness as those in which the living cells were normally measured. The difference was in fact so small that it was difficult to measure accurately, but it was found that the increase of apparent size of the scale in saline was, at the very most, $\frac{3}{4}$ per cent., and this factor can therefore safely be ignored when considering cell-size measurements which are accurate only to the nearest 7 per cent. approximately.

With the living cells it was necessary to find a standard mounting medium. The obvious choice would seem to be snail's blood, and this was in fact used initially, but it was difficult to obtain in quantity except by the crude method of decapitating the animal, with the consequence that it was more or less contaminated with mucus and digestive enzymes, and measurements obtained in it were rather variable. It was therefore decided to use saline media. Hédon-Fleig's (gastropod) saline gave consistent results, but as they were in every way identical with those given in a simple 0·7 per cent. NaCl solution (with the addition of 0·02 per cent. of $CaCl_2$), the latter was chosen in favour of any more elaborate mixture (fig. 1). (In addition, it was found that this simple saline solution was equally efficient as a medium for culturing snail amoebocytes from pieces of mantle tissue—the technique described by Gatenby and Hill (1934) and used by Crawford and Barer (1951) in their investigations on cell fixation. The cells migrated out of the tissue in exactly the same manner as in Hédon-Fleig's saline and the preparations were in all other respects indistinguishable in appearance.)

It was, of course, necessary to establish that 0·7 per cent. NaCl solution was in fact isotonic, and to do this it was thought advisable to find the limits of these cells' osmotic tolerance by measuring them in a range of salt solutions of different strengths. The results of this are shown in fig. 2. It will be seen that no significant shrinkage or swelling of either the whole cell or the nucleus occurs until the salinity reaches 0·9 per cent. and 0·4 per cent. respectively. Over a remarkably wide range, 0·5 per cent.–0·8 per cent., the modes remain constant, and these modes (15 eyepiece divisions (19 μ) for the whole cell, and 10 eyepiece divisions (12 μ) for the nucleus) were taken as datum lines from which shrinkages could be estimated. Incidentally, the wide tolerance is of interest in that it indicates a very efficient osmotic compensating mechanism inherent in the cell's metabolism.

The only other difficulty with the living cells was that they might be partially squashed by the pressure of the coverslip and appear larger than they really were; this was overcome by including a small piece of tissue debris in each preparation, thus raising the coverslip slightly at one point, and only cells in this region—which were often seen to be slowly moving and so were definitely free—were measured.

The living spermatocytes were nearly always practically spherical, but in the fixed preparations some degree of distortion often occurred, although the

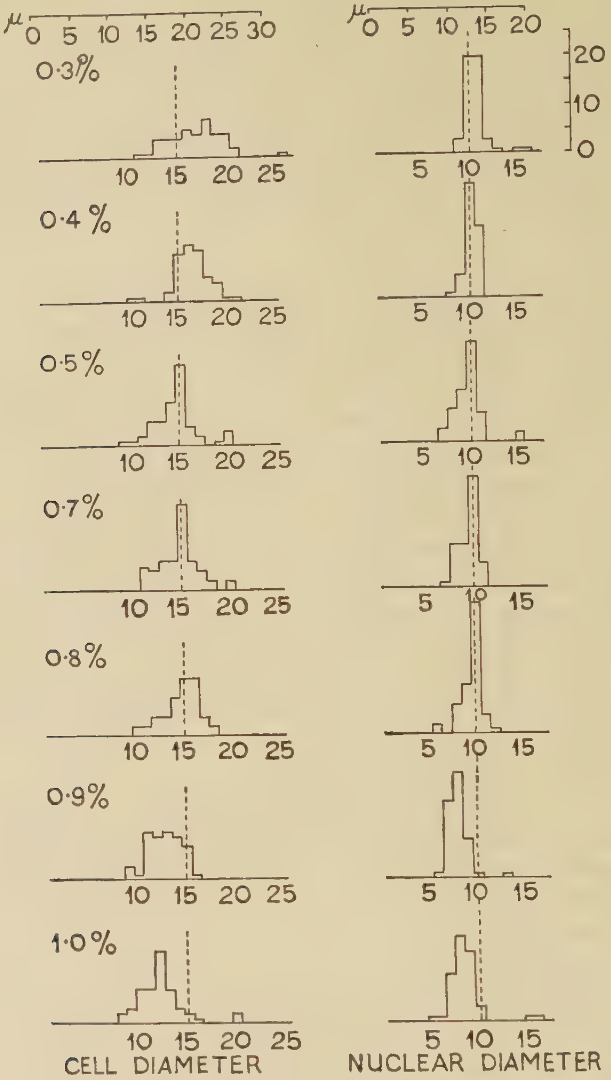

FIG. 2. Frequency polygons showing the size-distribution of living *Helix* primary spermat cytes in sodium chloride solutions at various concentrations. ($CaCl_2$ was present in each case 0·02 per cent.). Fifty cell measurements and fifty nuclear measurements from each preparatio

maximum and minimum diameters seldom differed by more than about per cent. Instead of measuring two diameters in these cases, the eyepiec micrometer scale was kept stationary throughout the examination, and dia meters were measured as the cells crossed this, regardless of where their axi lay. This was obviously justifiable with loose, unorientated cells.

There is one factor, however, that could affect orientation. It was Mr. H. Pusey who drew my attention to the possibility of distortion occurring fro the shearing action of the microtome blade. Aumonier (1938) found that sections of paraffin wax, without embedded material, of the same thickness (8 μ), were quite appreciably narrower than their block even after being expanded by flattening; and so, to check this, the following test was carried out on one of the blocks of fixed material. Several consecutive sections were cut, mounted on a slide, and flattened in the ordinary way. The block was then removed from the microtome chuck, and the back of the block was carefully pared down until it was of approximately the same thickness as a microscope slide. It was then mounted on a slide with the surface cut by the microtome uppermost. Its width in the direction of the sweep of the blade and the width of the embedded tissue were then measured with an eyepiece micrometer and a 3-inch objective. These dimensions were then compared with those of the adjacent sections, which were placed on two slides to bring them into the same plane of focus. The results, given in table 1, show that, although the block itself may be distorted by as much as 8 per cent. (which lies just within the limits of accuracy of the cell-measuring technique), the tissue only changes by some 6 per cent., and one can probably safely assume the cells themselves are even less affected by this factor. So this is really more troublesome to the histologist and the micro-anatomist than to the cytologist.

TABLE 1. *Dimensions of flattened paraffin-wax sections compared with that of the block from which they were taken, showing the distortion from the action of the microtome blade. (Block and sections measured with eyepiece micrometer and 3-inch objective.) Paraffin block was approximately one slide thick, and the focus of the microscope was left unchanged when examining sections which were mounted on the two slides. 8-μ sections. 52/54° M.P. wax. (The figures represent arbitrary units.)*

Paraffin block	6·3	Embedded tissue in block	3·7
Section *a* (proximal)	6·0	,, in section	3·5
b	5·8	,, ,,	3·5
c	5·8	,, ,,	3·5
d	5·7	,, ,,	3·5
A gap of three or four sections			
x	5·7		
y	5·7		
z	5·7		
Distortion of whole section $\frac{5}{63}$ = 8 per cent. approx.		Distortion of tissue $\frac{2}{37}$ = 6 per cent. approx.	

PRESENTATION OF THE RESULTS

Of the fixatives tried, nine were simple substances comprising nearly all the ingredients normally used as constituents of fixative mixtures, while eight were chosen from the most commonly used fixing mixtures. Seven of these—Aoyama, Bouin, Flemming, mercuric-acetic, mercuric-formol, Helly, and Zenker—may perhaps be classified as 'rational' fixing mixtures inasmuch as their action on protoplasm can, to a certain degree, be inferred from th chemical properties of their constituents; and they can be distinguished from the large numbers of (very often chemically unstable) mixtures in use, whos

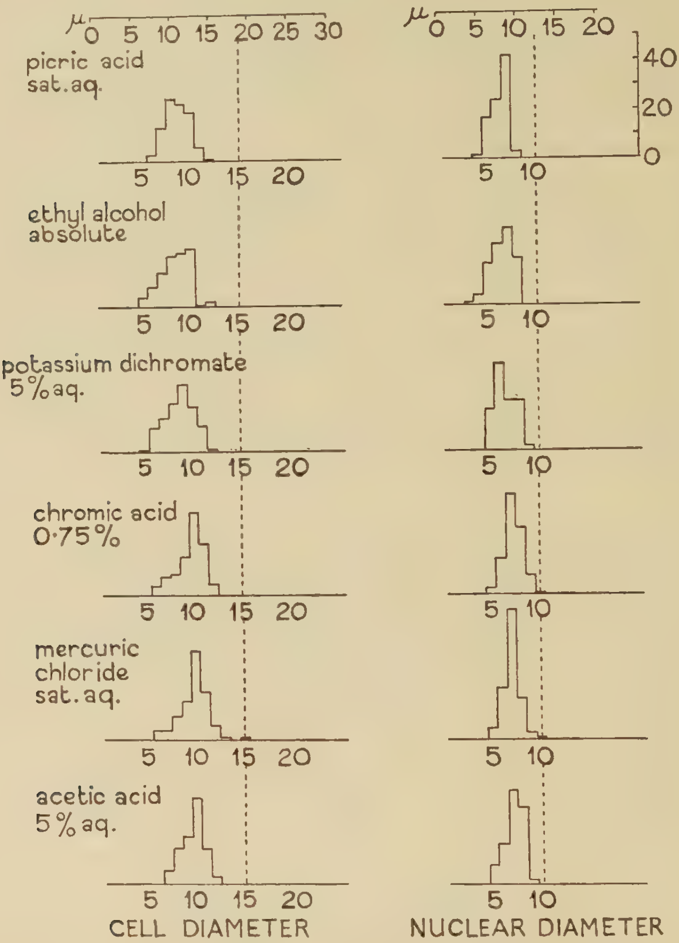

FIG. 3. Frequency polygons showing the size-distribution of *Helix* primary spermatocyte after fixation in various simple fixatives, embedding in paraffin, and mounting in Canada balsam. Measurements from two separate preparations are combined in each polygon (100 ce measurements and 100 nuclear measurements in all).

precise mode of action is by no means clear. It ought to be pointed out, however, that this designation need not necessarily imply condemnation in othe respects, and an 'irrational' fixing mixture included here (Sanfelice's fluid finds favour with many cytologists in giving clear-cut and consistent results In the case of those mixtures in which the proportions of the ingredient are sometimes varied, a note on the composition used in these experiment accompanies the histograms (figs. 3–5).

The frequency polygon serves as a concise method of presenting the whole collected data, that is to say the total cell-size distribution in each case. The amount the mode has shifted to the left of that of the living material (represented in every case by a dotted line) gives an indication of the shrinkage that has occurred. But as a basis for calculating actual figures for shrinkage the mode can be misleading, since the distributions are usually skew and may even be bimodal (as in the case of Sanfelice, fig. 5). It was therefore decided to use the median as the central tendency, and the medians (in μ) for the living material and the fixed are shown in tables 2 and 3 respectively.

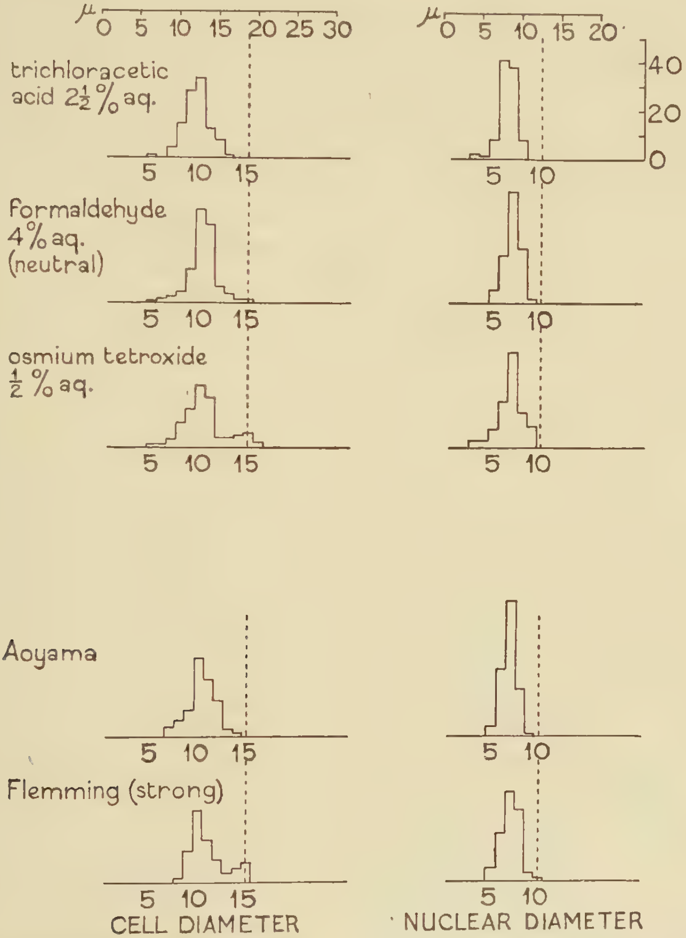

FIG. 4. Frequency polygons showing the size-distribution of *Helix* primary spermatocytes after fixation in various simple fixatives and fixing mixtures, embedding in paraffin, and mounting in Canada balsam. Measurements from two separate preparations are combined in each polygon (100 cell measurements and 100 nuclear measurements in all).

As was mentioned above, the work on the fixed material was done a second time, and in order to see just how closely the two sets of results corresponded the medians were calculated separately for each set of measurements, and th two compared. It was found that, with respect to whole cell-diameters, with n

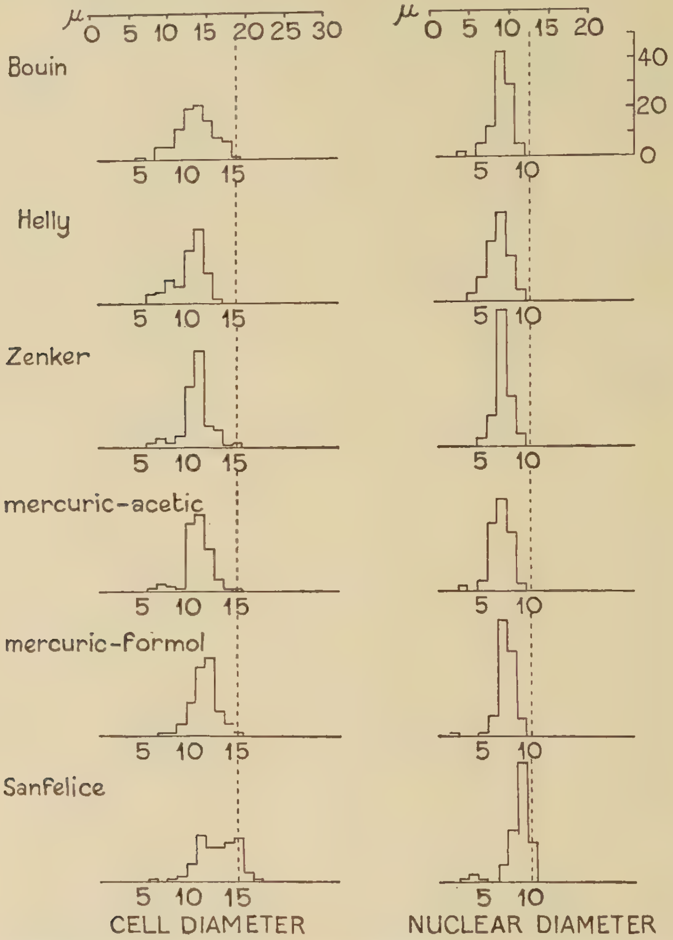

FIG. 5. Frequency polygons showing the size-distribution of *Helix* primary spermatocyte after fixation in various fixing mixtures, embedding in paraffin, and mounting in Canad balsam. Measurements from two separate preparations are combined in each polygon (100 cel measurements and 100 nuclear measurements in all).

Composition of the mercuric-acetic used:	
Mercuric chloride, sat. aq.	95 c.c.
Acetic acid (glacial)	5 c.c.

Composition of the mercuric-formol used:	
Mercuric chloride, sat. aq.	90 c.c.
Commercial formalin	10 c.c.

less than 11 out of the 17 fixatives tried the values obtained for the first an second experiments differed significantly. With respect to nuclear diameter the correspondence appeared to be rather closer, a significant difference occur ring in only seven cases; but as the nuclei are smaller, the measuring is here less critical.

This (at first sight) rather disturbing discovery might be held to cast doubt on the precision of the technique, although it is certainly true that considerable trouble was taken to make the conditions the same for the two sets of experiments. Alternatively, it might be taken to suggest that there may, after all, be an appreciable variation between individual snails with respect to their cell-size, but it can be seen in table 2 that the six living preparations measured differ from each other considerably less with respect to their medians than many of the fixed, and in only one case is this difference large enough to be significant.

TABLE 2. *Medians in μ (absolute size) of size-distributions of living cells in saline media.*

Snail number	*Cell-diameter*	*Nuclear diameter*
34 (0·7 per cent. NaCl) . .	18·4	12·1
28 ,, . .	18·9	12·2
51 ,, . .	18·7	. .
52 ,, . .	17·3	. .
28 (Hédon-Fleig's saline) . .	19·2	12·0
40 ,, . .	18·4	11·3
Combined medians . . .	*18·5*	*12·0*

On the other hand, when one considers the complexity and individual variability of the chemical substances in an animal cell and the continual changes they undergo during active metabolism, they can hardly be expected to react in precisely the same manner each time they are treated with a given reagent, like two simple chemicals in a test tube. From this viewpoint, it seems less unreasonable that there should be a certain variability of action when the same fixative is used more than once on the same kind of cell. On this contention one might expect a still greater variability to result when the fixative also is not a simple substance, and this is, indeed, suggested by the fact that in most cases the difference between the medians is greater in the case of the fixing mixtures than with the simple fixatives.

Nevertheless, it must be emphasized that although in the majority of cases these differences are large enough to be of statistical significance, they are not in any instance very large; and it is obvious that, even admitting the variability, *approximate* values for shrinkage can be assigned: the crucial question being the limit of accuracy that can be allowed.

On examination of table 3, it can be seen that, with respect to whole cell diameters, the fixatives can be classified into six fairly distinct groups, which can be arranged in order of decreasing shrinkage as follows:

The greatest shrinkage is shown after using certain simple fixatives:

1. Picric acid, ethyl alcohol, and potassium dichromate.
2. The remaining simple fixatives except neutral formalin and osmium tetroxide.

3. Neutral formalin and Aoyama's fluid.
4. A markedly variable group comprising osmium tetroxide, and all the 'rational' fixing mixtures except mercuric-formol.
5. Mercuric-formol.
6. Sanfelice's fluid, which very noticeably shrinks least of all.

TABLE 3. *Medians in μ (absolute size) of size distributions of cells in fixed preparations*

Fixative	*Cell-diameters*		*Nuclear diameters*	
	Expt. 1	*Expt. 2*	*Expt. 1*	*Expt. 2*
picric acid	11·5	10·2 S	8·5	7·4 S
ethyl alcohol	10·2	11·2 S	8·1	8·4
potassium dichromate	10·8	11·9 S	7·4	9·1 S
chromic acid	12·5	12·1	9·9	8·5 S
mercuric chloride	12·6	12·1	8·7	8·5
acetic acid	12·5	11·8 S	8·6	9·1
trichloracetic acid	12·2	11·9	8·2	7·9
neutral formalin	13·4	12·5	8·7	8·7
osmium tetroxide	12·4	14·6 S	8·7	8·3
Aoyama	12·6	13·1	8·0	9·0 S
Flemming	12·5	14·9 S	8·4	9·4 S
Bouin	12·6	14·6 S	8·9	9·0
Helly	12·1	13·8 S	7·6	9·4 S
Zenker	12·8	13·9 S	8·6	9·1
mercuric-acetic	13·2	14·4 S	8·1	9·1 S
mercuric-formol	14·0	14·8	9·3	9·1
Sanfelice	16·6	15·5 S	11·1	10·9

The letter S in the third and fifth columns indicates a significant difference between the medians given by the two sets of measurements.

With respect to nuclear diameters, there is considerable overlap, and classification is a rather more arbitrary affair, but five main groups can be made out which broadly correspond to those for whole cell-size.

Tables 4 and 5 show percentage shrinkages worked out for each of these groups, with the approximate error in each case. The error here is not solely defined by the maximum difference between the medians but is taken to extend beyond them by at least 1 per cent. in each direction. While no claims are made that they are above criticism, the values given are probably essentially correct.

It must be stressed that the shrinkage referred to here is the final shrinkage due to *all* the treatment received by the cell up to and including mounting in balsam. Some fixatives that do not shrink (e.g. acetic acid) permit shrinkage by subsequent procedures.

CONCLUSIONS

For reasons already fully discussed, it is certainly inadvisable to attempt to relate the shrinkages found to the chemical properties of the fixatives, and it

TABLE 4. *Approximate shrinkages of whole cells (per cent.)*

Fixative	*Per cent. shrinkage in diameter*	*Per cent. shrinkage in volume*
(1) picric acid ethyl alcohol potassium dichromate	40±6	78±6
(2) chromic acid mercuric chloride acetic acid trichloracetic acid	34±4	71±5
(3) neutral formalin Aoyama	30±4	66±6
(4) osmium tetroxide Zenker Flemming Bouin Helly mercuric-acetic	27±9	61±14
(5) mercuric-formol	22±4	53±7
(6) Sanfelice	13±5	34±11

TABLE 5. *Approximate shrinkages of cell nuclei (per cent.)*

Fixative	*Per cent. shrinkage in diameter*	*Per cent. shrinkage in volume*
(1) picric acid ethyl alcohol trichloracetic acid	34±6	71±8
(2) potassium dichromate	31±9	67±13
(3) osmium tetroxide mercuric chloride Aoyama Helly mercuric-acetic	28±9	63±14
(4) chromic acid neutral formalin acetic acid Zenker Flemming Bouin mercuric-formol	23±7	54±12
(5) Sanfelice	9±3	25±7

would be unwise to try to draw too many conclusions. Nevertheless, a certain number of points seem to have emerged during the course of the work which can be summarized briefly as follows:

(1) The living snail primary spermatocytes will tolerate quite a wide range of salinity (0·5 per cent. NaCl–0·8 per cent. NaCl inclusive) without any detectable change in size.

(2) Almost without exception, cells embedded in paraffin wax are shrunk more after being fixed with simple fixatives than when fixed with fixing mixtures.

(3) Nuclear shrinkage seems, for the most part, to be fairly consistently related to whole cell shrinkage, but the cytoplasm nearly always shrinks more than the nucleus.

(4) Sanfelice's fluid is remarkable in giving considerably less shrinkage than any other reagent. The mixture contains acetic acid, which, used alone, often initially swells cells, and it might be thought that its use here in conjunction with a protein precipitant (chromic acid) enables the cells to be fixed in a distended condition and shrink relatively less in the subsequent embedding; but this cannot be the whole story, since mercuric-acetic, Zenker's fluid, &c., also contain acetic acid and a protein precipitant, and are in no way remarkable in this respect.

(5) Excepting Sanfelice's fluid, there is no very great difference between the shrinkages given by any of the reagents here tried; and the cytologist who, as a rough approximation, assumes that the cells in a paraffin-wax preparation have shrunk linearly by about *one third* is unlikely to be very far wrong.

This last conclusion is certainly the most important since, in emphasizing the general similarity between all the preparations, it suggests that of the two factors responsible for cell shrinkage mentioned in the introduction—the fixation and the subsequent embedding—the embedding process, which was kept as an invariable in these experiments, is a very important factor in determining the final cell size; and in many cases the individual fixatives may play only a subordinate role. All the reagents used in paraffin-wax embedding, alcohol, xylene or toluene, and molten paraffin-wax tend to shrink cells and, generally speaking, the effect of these reagents together with the fixative is to reduce the *volume* of the cell to about 30 per cent. of what it was in life.

It is necessary to add one final word of caution: all the work described was done with very small pieces of loosely organized tissue, and the reagents, to all practical intents and purposes, had immediate access to every cell. It is by no means certain whether the same results would be obtained in the case of cells deeply embedded in relatively large pieces of compact organs, such as often form the basis of standard histological preparations (as opposed to cytological preparations). This particularly applies in the case of fixing mixtures, for it is known that one ingredient often penetrates tissues faster than another.

ACKNOWLEDGEMENTS

In addition to those whose help has been more conveniently acknowledged in the text of this paper, I wish to thank Dr. J. R. Baker for his advice on the all-important question of the choice of material for the work, for his encouragement and very helpful supervision, and for reading and correcting my manuscript; and also Dr. D. I. Finney of the staff of the Bureau of Design and

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I also wish to thank Mrs. Seldon and Miss Court for their help in preparing my manuscript and figures.

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Cytological Studies of the Acinar Cells of the Pancreas of the Mouse

Part I. The Formation of Neutral Red Granules

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With one plate (fig. 1)

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SUMMARY

1. It has been found that during a 12–15 hour period after the subcutaneous injection of mice with a predetermined 'optimum' dose of neutral red, there occurs a definite series of changes in the pancreas acinar cell in which neutral red granules are formed as separate bodies, which subsequently come together as aggregates and then disappear from the cell.

2. The cell is not damaged by the cycle of events just described. The response to the presence of neutral red appears to be physiological, not pathological.

3. Evidence is brought forward showing that neutral red granules and aggregates are not vacuoles that pre-existed in the normal cell and were subsequently stained: they are new formations produced by the presence of neutral red in the cell.

4. Little more than one-quarter of the neutral red injected is excreted in unchanged form.

INTRODUCTION

IN undertaking a cytological study of the exocrine cell of the pancreas of the mouse, the writer had occasion to use neutral red as a vital dye. Neutral red has been much used in attempts to elucidate the true structure of the so-called Golgi apparatus. Its use has given rise to controversy among cytologists. Some have supposed that the dye colours pre-existing vacuoles or 'granules' in the cytoplasm, generally situated in or close to the part of the cell in which the Golgi apparatus is seen in fixed preparations, while others have claimed that the dye does not stain pre-existing objects but *causes* the production of vacuoles or granules as new formations not represented in the

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normal cell. Different authors, describing the same kind of cell, often give different information as to the number, size, position, and state of separation or aggregation of the neutral red granules.

It occurred to the writer that many of the discrepancies in the accounts given by other investigators might be due to the lack of any systematic attempt to discover the *dosage* of the dye that would give the most valuable information. Those who have studied the pancreas of the mouse have injected widely different amounts. Thus Covell (1928) gave a single injection of 1·5 ml. of a 1 per cent. solution of the dye; Chlopin (1927) gave repeated injections; Beams (1930) gave up to 12 ml. of a saturated solution. Some authors injected the dye subcutaneously, others intraperitoneally, while others again immersed pieces of pancreatic tissue in a solution of the dye. Not much attention has usually been paid to the exact period of time elapsing between injection and microscopical examination.

The writer decided to try to standardize what will here be called an optimum dose, that is to say, a dose that would be sufficient to show neutral red granules clearly, but would not cause gross pathological changes nor prevent the cell from returning in the course of time to a perfectly normal condition. He also decided to make his observations at precisely measured times after injection, so as to find whether there was any regularly occurring cycle in the appearance, growth, and disappearance of the granules.

MATERIAL AND METHODS

Adult male mice weighing 25 to 35 gm. were used exclusively in this study. Approximately 200 mice from several different lots were studied.

Neutral red chloride, the only form of neutral red tried, was obtained from four different makers. They were: British Drug Houses, Ltd. (batch numbers 673505/510228 and 672311/510615); Hopkin and Williams Ltd. (batch number 31517); G. T. Gurr (batch number 04955); and Edward Gurr Ltd. (batch number 352/1). The results with all of these were essentially the same.

The fluid in which the neutral red powder was dissolved for injection did not appear to influence the results materially. Both normal saline and distilled water were used. The latter, however, on the whole gave the best results.

Mice were always taken from a feeding stock cage, injected subcutaneously on the sides or back, and killed by a blow on the head. The site of injection chosen would seem to be of some importance, for when injection over the abdomen is attempted the abdominal wall is easily punctured, and one cannot always be certain that the injection has not been given intraperitoneally. It may be said in passing that the practice of introducing the neutral red subcutaneously obviates the criticism that intraperitoneal injection of distilled water causes abnormal changes in the pancreas (Gatenby, 1931).

The method of studying the fresh pancreas was as follows. Immediately after the removal of the pancreas from the body, pieces measuring about 0·5 mm. to 1 mm. in greatest dimension were cut off from several regions and placed on a slide. A drop of normal saline was added and a coverslip applied. With pieces of tissue of this size, the weight of a No. 1 coverslip is sufficient to flatten the tissue without undue crushing or distortion. Upon examination with the microscope the specimen appears to be about the thickness of 3 to 5 acini. This thickness, admittedly, is not ideal and to some extent spoils the illumination; however, the conditions are adequate for good general examination, and the thin edge of the tissue is easily studied.

The dose of neutral red finally considered as optimum was approximately 0·0002 gm. per gramme of mouse body-weight. This is conveniently administered in a 1 per cent. solution, and it is sufficient for results that mice weighing 25 to 29 gm. receive 0·6 ml. and those weighing 30 to 34 gm. 0·7 ml., &c. The possibility must be kept in mind that a different batch of neutral red may require its own standardization. However, in view of the similar findings derived from the five batches tried in this study, it seems unlikely that the variation from 0·0002 gm. per gramme of mouse body-weight will be very great.

THE NEUTRAL RED GRANULE CYCLE

Fifteen minutes after the subcutaneous injection of the optimum dose of neutral red, the only change observed in the pancreas acinar cell is one of colouring. The cytoplasm, which is normally colourless, is now homogeneously light pink. Neutral red granules are not identified at this stage.

Half to three-quarters of an hour after injection, the cytoplasm is slightly darker pink and there are four to six small, separate, spherical, reddish granules about 0·7 μ in diameter scattered in the regions of the cytoplasm just basal to the zymogen granules and for a short distance along the sides of the nucleus. The neutral red granule is sharply demarcated from the surrounding, homogeneous, pale pink cytoplasm. It resembles very closely a drop of coloured oil in water. It is not birefringent. It is too small to permit one to draw detailed conclusions about its finer structure, and one can only say that it appears to consist of a fluid immiscible with the ground cytoplasm.

During the course of the next half-hour to three-quarters of an hour, there is a gradual increase in the number of the neutral red granules until there are 8–12 of them. During this period it is observed that some of the neutral red granules no longer occur as separate bodies but apparently have come together to form clumps or aggregates of two or three granules.

During the ensuing periods up to about 8 hours after injection, the process of aggregation becomes more marked (see fig. 1). Fewer single granules are seen and each aggregate appears to consist of about 4–8 granules; these are bound together by some unknown force, but do not fuse. Each aggregate resembles a small bunch of grapes. It is often not possible to count the number of granules in each aggregate with certainty, as some of them tend to obscure others. At late stages of aggregation each clump of granules tends to be somewhat elongated. The position of the neutral red granule aggregates is similar to that first observed for the single granules—in the region just basal to the zymogen granules and along the sides of the nucleus. They are not observed mixed amongst the zymogen granules or immediately adjacent to the base of the cell.

Additional changes in the aggregates during the latter stages of development include an increase in intensity of staining and some further increase in size and number. At the time of their maximum development there may be 15–20 aggregates, each 2·5 to 3 μ in greatest dimension.

After about 8 hours there is a gradual decrease in number and size of the aggregates and discrete granules until at 12 to 15 hours after injection of neutral red, the cytoplasm is free of both elements and normal in appearance.

It should be added that if a larger dose of neutral red is injected, the only variation from the sequence of events just described is that the granules and aggregates are formed more quickly after injection and persist longer.

It is concluded, therefore, from the foregoing results that there is, as had been anticipated, a definite series of changes in the mouse pancreas acinar cell by which the neutral red granules appear and later disappear after the subcutaneous injection of a predetermined optimum dose of neutral red.

CRITIQUE OF THE METHOD USED IN STUDYING THE GRANULES

In any experiment in which a foreign substance is introduced into an animal, the possibility that the functioning of certain cells will be disturbed by that foreign substance must receive first consideration. Further, the possibility that what one observes in the cell after the introduction of a foreign material represents not the normal workings of the cell within the limits of its physiological potentialities but manifestations of disturbed functioning, must be kept in mind.

These were points of major consideration in the determination of the optimum dose of neutral red. In fact, the optimum dose finally decided upon was a direct result of such considerations.

Previous mention has been made of the fact that if very small doses of neutral red were injected the neutral red granules did not develop, but if very large doses such as 1 ml. of a saturated solution were given, pathological changes occurred. These changes, interpreted as pathological, were observed several hours after the administration of neutral red and took the form of large reddish vacuoles measuring up to 4 μ in diameter, filling the cytoplasm.

FIG. 1 (plate). Photomicrographs of living acinar cells of the pancreas of the mouse, 5 hours after the subcutaneous injection of the optimum dose of neutral red. The specimen consisted of a piece of tissue about 0·5 mm. across, placed in normal saline on a microscopical slide. (The neutral red granules of an acinus cannot all be in focus at the same time.)

A shows the number and size of the neutral red granule aggregates and their position in relation to the zymogen granules. The neutral red granules appear black in the photograph, the zymogen granules white.

B shows the position of the neutral red granule aggregates in the cell: they are basal to the zymogen granules. The apical borders of three cells are in focus in the centre of the acinus (at the site of the potential lumen).

C shows the irregular, finely nodular structure of the neutral red granule aggregates.

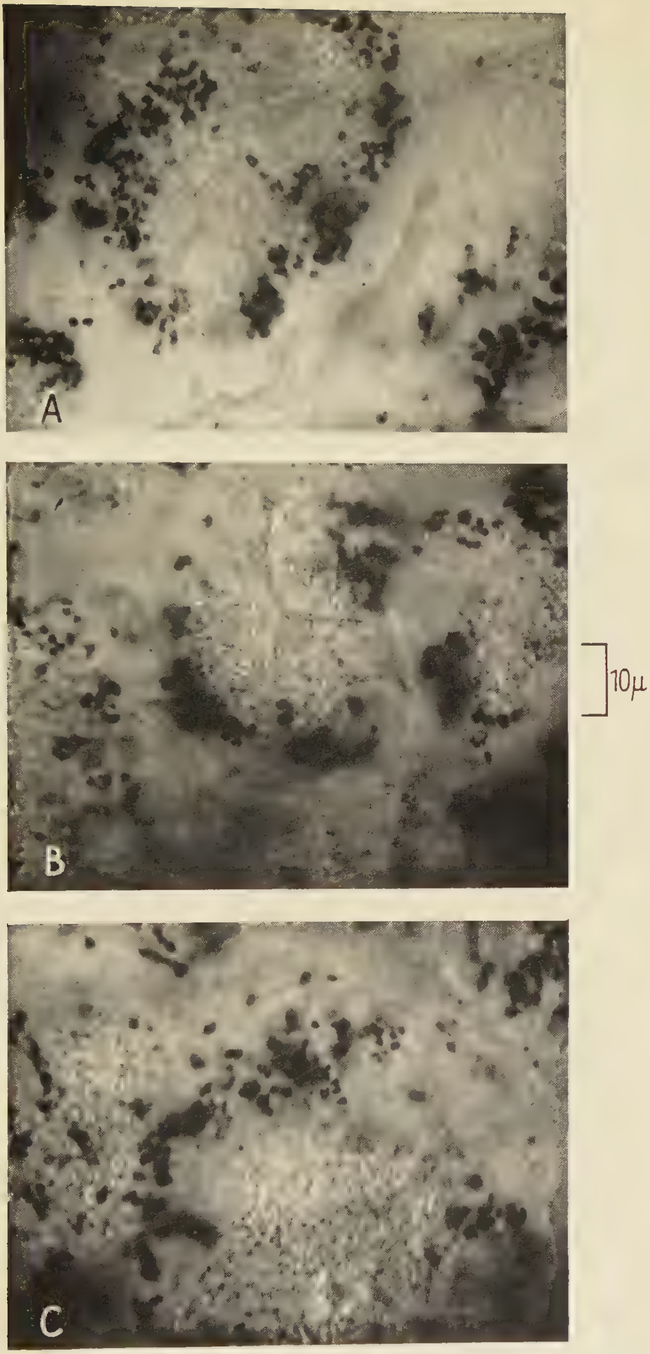

Fig. 1
W. S. MORGAN

Were they not coloured, they would strongly suggest a state of pathological change in the cell commonly known as hydropic degeneration, that is, extensive vacuolization. The task at hand, then, was to find the dose of neutral red intermediate between these two extremes in which the neutral red granules could be observed under conditions in which the physiological stability of the cell was preserved. With this end in view, the following points were taken into consideration:

(1) The volume of the neutral red solution injected should not be excessively large in proportion to the size and weight of the animal and it would be an advantage if that volume were one which could be conveniently administered.

(2) The dose of neutral red should not be one which would make the animal obviously ill. The mice remained alert and active after the optimum dose had been injected. Such was not the case when large doses were administered, for the animals became lethargic and weak. It should be recorded, however, that when the optimum dose was given, the animals seemed disinterested in food until some 12 to 15 hours later, when the pancreas was cleared of neutral red.

(3) It was felt that some measure of safety could be afforded the cell if the dose was one in which it required several hours before maximum development of the neutral red granules was attained. It was considered that in this way the cell could perhaps gradually adjust itself to the presence of a foreign material, in contrast to a dose the size of which would cause sudden or maximum changes within the first hour or two after injection.

(4) The dose of neutral red should be one in which the complete cycle of neutral red granule formation and disappearance could be followed, but one by which neutral red did not remain in the tissues for an excessively long period. The possibility was entertained, but without evidence, that the presence of neutral red for an extended period might in some way injure the cell, and it was known that within 24 hours, if no food was taken, the general metabolism of the mouse would be upset as evidenced by the development of fatty changes in the liver. Accordingly, it was felt that 12 to 15 hours represented the safest length of time during which the neutral red granules could be studied.

(5) The dose should be one which did not occasion either immediate or late harmful effects. The immediate effects of the optimum dose have been discussed above (2). In an attempt to discover the presence of any late effects, animals were observed for a month after the injection of neutral red. They were completely normal in all respects.

The foregoing may be regarded as reasonable measures for preserving the normal functioning of the cell, but it was felt that evidence of a more objective nature should be secured indicating to what degree, if any, the general physical and chemical balance of the cell had been disturbed by the presence of neutral red.

It is generally agreed that mitochondria are very susceptible to alterations in cytoplasmic conditions. Thus, after exposure of pancreatic tissue to certain fixatives, or when mitochondrial fractions from homogenized pancreas are suspended in hypotonic solutions, the mitochondria fail to retain their natural rod-like structure but assume spherical, globular, or gourd-like shapes. Such forms are considered by cytologists to be abnormal.

It was therefore concluded that a study of the mitochondria during the several stages of neutral red granule formation after the injection of neutral red, would provide information as to whether the precautionary measures previously discussed were of value and whether the optimum dose of neutral red caused any serious alteration in the normal functioning of the pancreas acinar cell.

Two methods of approach to this problem were employed, namely, the classical Altmann technique for showing mitochondria, and the immersion of bits of fresh pancreas for 15 minutes in a 1:500 solution of Janus green. Both methods showed apparently normal rod-like mitochondria in the pancreas after injection of neutral red; the mitochondria resembled those in the uninjected control.

It is concluded from these findings that the administration of neutral red in the dosage finally determined as optimum does not upset the normal dynamics of the pancreas acinar cell to a degree which can, at the present time, be shown cytologically. Therefore, it is submitted that until the time when more sensitive methods for examination of cellular stability are available, the fairest interpretation of the present results is that the cycle following the injection of the optimum dose of neutral red in which neutral red granules form in and disappear from the cell, represents, not necessarily the normal functioning of the cell, but a process within the physiological capabilities of the cell.

THE ORIGIN AND NATURE OF NEUTRAL RED GRANULES

It is necessary first to ask whether the neutral red granules and aggregates represent pre-existing vacuoles in the cell which are coloured when in contact with the dye, or whether they are new formations, objects not normally present in that form and position in the cell, but produced by the entry of neutral red into the cell.

It is important that this question should be asked about each type of cell whenever neutral red is used, for it is known that in some cells, such as ganglion or liver cells, there are vacuoles readily visible in the fresh, unstained state as clear, colourless vacuoles, which become red after the immersion of the tissue in a solution of neutral red; because of their shape and size they qualify for the name 'neutral red granules'. In other tissues, in which red granules develop after injection of neutral red, similar vacuoles are not apparent in the unstained cell, and one is left to conclude that in the second type of tissue, the neutral red granules clearly visible after the injection of neutral red are new formations produced by the presence of neutral red in the cell. To these two must be added the third possibility that the neutra red granules seen after injection of neutral red represent a combination of both pre-existing and newly formed vacuoles.

It is the author's opinion that the mouse pancreas acinar cell falls in the second group, i.e. that the neutral red granules are new formations. This conclusion is based on the following studies and considerations:

(1) The positions of the neutral red granules and aggregates have from the study of the neutral red granule cycle been established as being confined to the regions just basal to the zymogen granules and for a short distance along the sides of the nucleus. Further, these are the only sites in which the neutral red granules are seen; they are not observed mixed amongst the zymogen granules, or basally in the cell immediately adjacent to the basement membrane. Routine microscopic examination of these regions of the cell in tiny bits of normal unstained pancreas discloses a homogeneous cytoplasm. This is 'empty' except for the ends of mitochondria, and sharply demarcated from the prominent zymogen granules.

(2) Pre-existing vacuoles are readily visible by ordinary microscopy in the liver cell, and upon staining with neutral red certain ones are not unlike the neutral red granules seen in the pancreas. The zymogen granules in the pancreas have the appearance of vacuoles, and they are the most prominent component in the unstained living cell. The author finds it impossible, therefore, to accept the statement that there are vacuoles normally present in the unstained pancreas acinar cell in the number and of the size of the neutral red granules and aggregates. As previously described, the neutral red granules and aggregates in the advanced stage of development occupy a major portion of the cytoplasm just beneath the zymogen granules and the aggregates measure up to 2·3 μ in greatest dimension. Vacuolar structures of this size should be easily visible.

(3) The application of phase-contrast microscopy was found to be impossible when 1 mm. pieces of tissue were used, because the thickness of the specimen caused excessive diffusion of the light. In subsequent attempts to secure specimens of pancreas suitable for phase study, tissue was homogenized in an all-glass tube, and although small clumps of acinar cells were obtained, these showed too much distortion of cellular architecture to be considered of value. Finally, the method of teasing bits of pancreatic tissue in normal saline with sharp needles provided suitable preparations for study. Examination of these cells failed to show vacuoles or aggregations of vacuoles above or beside the nucleus, that is to say in the positions occupied by the neutral red granules or aggregates after the administration of neutral red.

(4) There is still another method by which the presence of vacuoles stainable with neutral red can be ascertained. This is the commonly used procedure of simply immersing bits of tissue in a solution of neutral red. Presumably, as in the liver and ganglion cells in which pre-existing vacuoles may be identified and which are coloured by neutral red, similar vacuoles if present in the pancreas should become stained and apparent after immersion of this tissue in neutral red. Accordingly, two experiments were carried out. Small pieces of pancreas measuring 0·5 mm. to 1 mm. were divided, some being placed in a 0·001 per cent. solution of neutral red in normal saline,

others in a 0·001 per cent. solution of neutral red in distilled water for periods up to 3 hours.

The results of this study showed that in the specimens of pancreas immersed in the solution of neutral red in distilled water, although there was extensive diffusion with resulting pinking of the tissue, this colouring was homogeneous, affecting all the cell components: at no time were neutral red granules observed. In contrast, the bits of pancreas which were treated similarly in a solution of neutral red in normal saline showed many neutral red granules. Although the organization of these was never as neat as in tissue from injected animals, their character and distribution were interesting.

In the inner portions of the specimen, the neutral red granules were small and separate, occupying roughly the area of the cytoplasm just basal to the zymogen granules and to the sides of the nucleus. In areas lying roughly intermediately between the inner portion and the edge of the specimen, separate granules and clumps of granules similar to aggregates were observed. At the very edge of the specimen the elements stained with neutral red were in the form of large vacuoles scattered throughout the cell. These last were in no way dis-similar to the structures previously described as pathological and observed in the pancreas of an animal after injection with a large dose of neutral red.

These findings are interpreted in the following manner:

(1) It is assumed that the cells placed in a distilled water solution of neutral red did not live long. If pre-existing vacuoles stainable with neutral red were present they should have been apparent, since there was ample diffusion of neutral red into the tissue.

(2) On the other hand, it is assumed that the cells placed in neutral red in normal saline lived for a considerable period and because of this, conditions were to some extent comparable to those in the pancreas of the living animal. It is therefore concluded that the living cell detached from the animal in contact with neutral red has the same capacity for neutral red granule formation as the acinar cell in the animal injected with neutral red.

In addition to those workers who feel that the neutral red granules represent pre-existing vacuoles present in the normal cell, there are those who hold that still another component of the cell forms the neutral red granules. Bensley (1911) and Gatenby (1931) have stated that the neutral red granules are simply the prozymogen or immature zymogen granules which have been stained by neutral red. In view of this possibility it was felt necessary to repeat their studies by methods similar in principle to those they adopted. Mice were placed in metal cages without food (but with water) for a period of 24 hours; both starved and control (feeding) animals were then given a subcutaneous injection of the optimum dose of neutral red. Six hours later, food still being withheld from the experimental animals, both groups of animals were killed and the pancreas examined microscopically.

It was the contention of Bensley and of Gatenby that in the 24-hour period in which no food was ingested, there would be a maturation of all the prozymogen granules in the pancreas, leaving none to be stained by neutral red. In their experiments (Bensley using guinea pigs and Gatenby the newt *Triturus* (= *Diemyctalus*), they reported that in the starved pancreas neutral red granules were not present whereas they were observed in the pancreas of a feeding animal. They concluded from this that the neutral red granules were actually the prozymogen granules.

We were unable to confirm these findings of Bensley and Gatenby in our experiments with the mouse, the pancreas of both groups showing large numbers of neutral red granule aggregates as they had been seen many times before during the study of the neutral red granule cycle.

Although the possibility of a species-difference must be recognized, we have concluded from our experiments that in the mouse it is not the prozymogen granules that form the neutral red granules.

The contention of Hirsch (1939) that at least the Golgi 'pre-substance' is stainable with neutral red, is one which the present author does not feel qualified to discuss. Although 8 months of the writer's time was devoted to a study of the secretion cycle in the pancreas, his results in this line were unsatisfactory, and definite conclusions concerning the Golgi system during the secretion cycle as described by Hirsch were never reached. Although a definite statement must be deferred for the present, the author is strongly inclined to believe from data which will be presented later, that the neutral red granules observed in the present study are not pre-substance stained by neutral red.

There is one final possible source for vacuoles, which, to the author's knowledge, has not been previously advanced. It is conceivable that there exist in the pancreas cell small granules which cannot be differentiated from the zymogen granules, and that these, when in contact with neutral red, go on to form the neutral red granules. In such a hypothesis, however, one would expect to observe neutral red granules mixed amongst the zymogen granules, but they are not. Further, this hypothesis would imply a movement of such granules from the region of the zymogen granules to those parts of the cell where the neutral red granules and aggregates are found, as previously described. At no time was evidence encountered to support such a possibility.

It is concluded, then, from the foregoing considerations and experiments that the neutral red granules as observed after the injection of neutral red, are not stained pre-existing vacuoles normally present in the cell, but that they are new formations produced by the entrance of neutral red into the cell.

It has been said by some investigators (Covell, 1928; Beams, 1930), that neutral red granules are seen scattered amongst the zymogen granules, and it has been suggested that the neutral red granules are extruded into the lumen of the acinus along with the zymogen granules, and in that way the neutral red is removed from the cell.

The author does not consider that neutral red granules are present in the region of the zymogen granules. On numerous occasions during the study of the pancreases of animals injected with neutral red, the initial impression was that the neutral red granules were mixed amongst the zymogen granules; but when the object was carefully focused so that the majority of the zymogen granules of a single cell were sharply in view, no neutral red granules could be identified. This observation suggests that when the zymogen granules of one acinus are not in sharp focus, the neutral red granules which appear to be among the zymogen granules actually belong to an over- or underlying cell of another acinus. This may have been the mistake made by previous investigators who claimed that neutral red granules were observed in the region of the zymogen granules.

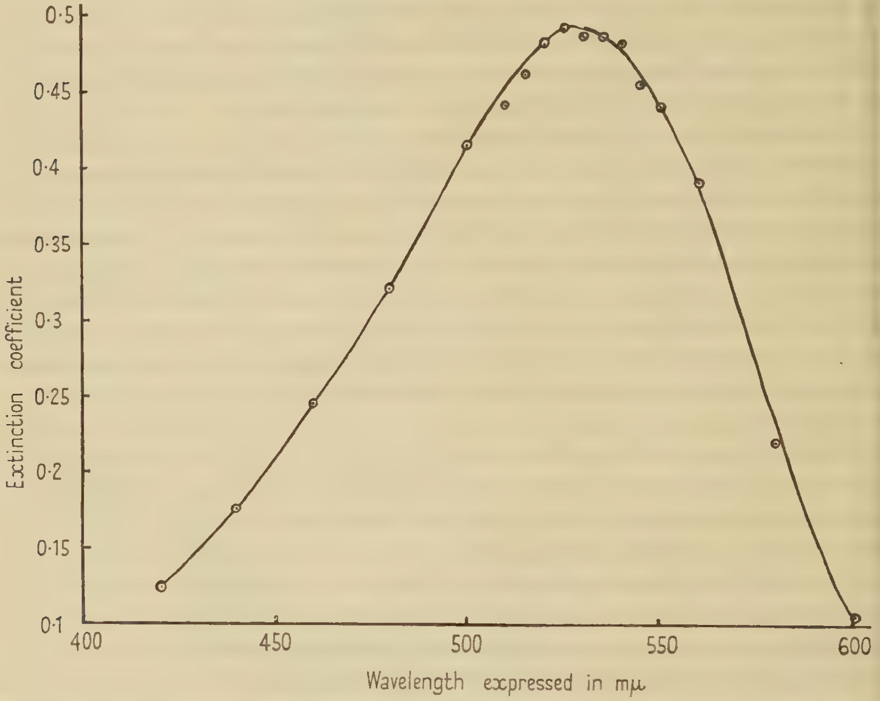

FIG. 2. Graph showing the absorption of light of different wave-lengths by an acid solution of neutral red.

THE EXCRETION OF NEUTRAL RED

It was observed during previous experiments that after the injection of neutral red, the urine was red not only during the 12- to 15-hour neutral red granule cycle observed in the pancreas, but also until about 48 hours after injection. It was concluded, therefore, that urinary excretion was at least one method by which neutral red was removed from the body, and it was felt that some information concerning the fate of the neutral red in the body might be revealed by a comparative study of the amount of neutral red injected with that excreted in the urine. It was felt that if all or nearly all the neutral red injected were recovered from the urine, this result would prove that the neutral red is unchanged when in the neutral red granules. If, on the contrary, much less than the total amount were recovered, this would suggest that the neutral red might be broken down while in the granules. It was recognized, of course, that the breakdown might occur in some organ other than the pancreas (e.g. the liver).

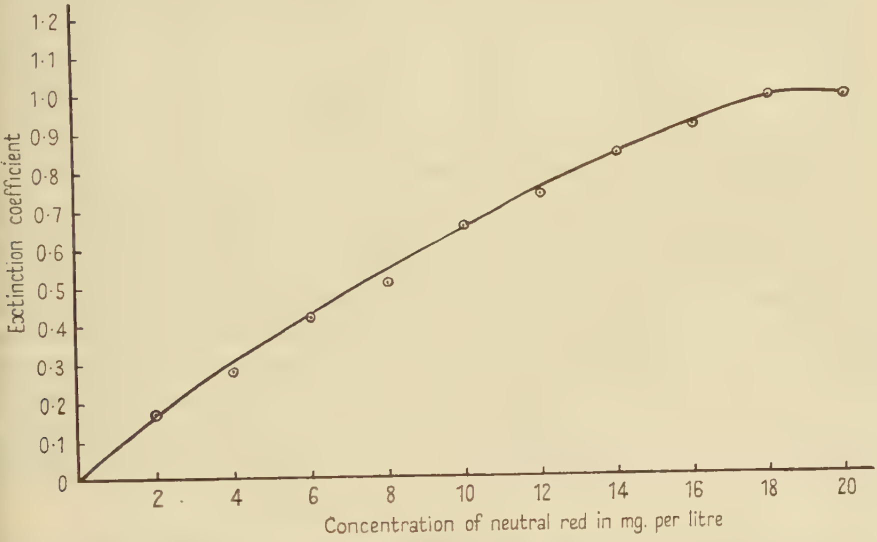

FIG. 3. Graph showing the absorption of light of wave-length 525 mμ by an acid solution of neutral red at different concentrations

Below pH 6 the dye is red. The extinction coefficient at various wave-lengths, at this pH, is shown in fig. 2. It will be noticed that the maximum absorption is at a wave-length of about 525 mμ. In fig. 3 the extinction coefficient with light of this wave-length is plotted against various concentrations of neutral red in aqueous solution at pH 6. This graph may be used to determine the concentration of neutral red in any solution of the dye. It is only necessary to measure the extinction coefficient of the specimen of unknown concentration, and the concentration of the dye can then be read off from the graph.

This method was used to measure the amount of neutral red in the urine of injected mice. Groups of two or three adult mice of similar weights were injected with the optimum dose of neutral red and placed in metabolism cages, from which all urine was collected. The urine specimens were made up to a volume of 250 or 500 c.c. according to the amount of washing of the cage necessary to recover all the urine. The specimens were acidified and colorimetric readings were taken. This was done at 24 hours and 48 hours after injection. Longer periods than this were not necessary, because earlier experiments had shown that after 48 hours too little neutral red was excreted to give colorimetric readings.

Table 1 shows the results of these studies.

TABLE 1

Expt. No.	*No. of animals*	*Amount injected (mg.)*	*Amount recovered in 1st 24-hr. period (mg.)*	*Amount recovered in 2nd 24-hr. period (mg.)*	*Total amount recovered in 48 hrs. (mg.)*	*Percentage recovered in urine*
1	3	21	5·625	0·36	5·99	28·0%
2	2	16	4·30	0·23	4·53	28·0%
3	3	24	5·45	1·20	6·65	27·7%
4	3	21	5·10	0·55	5·65	26·9%
5	3	21	5·45	0·34	5·79	27·6%
6	3	21	5·75	0·69	6·44	30·7%

These results indicate that only a little more than one-quarter of the neutral red injected is excreted in the urine as neutral red. After 48 hours no part of the body appears to be coloured red, and it is therefore unlikely that storage of unaltered neutral red occurs. Since examination of the faeces of these animals failed to show the gastro-intestinal tract as the route by which neutral red is removed from the body, the two main avenues by which foreign or toxic substances are discarded from the body have been excluded. It is therefore concluded that the major portion of a given dose of neutral red is altered within the animal body. The exact site and the method by which this is achieved have not been determined.

COMMENT

It will be observed that no attempt is made in the present study to relate findings to function in the pancreas, although several interesting possibilities of what is going on in the neutral red granule are considered. In what ways, if any, these may reflect the normal everyday secretion processes in the pancreas acinar cell are additional problems to be explored. At this point it should be said that in older research with neutral red the practice of assuming that findings with one type of cell were true for others was a fundamental error and contributed heavily to the confusion associated with the Golgi problem. The author wishes to emphasize that the studies reported here were confined to the pancreas acinar cell of the mouse. Whether similar neutral red granule cycles may be shown in other tissues or other species must await further research.

ACKNOWLEDGEMENTS

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Studies of the Mesogloea of Coelenterates

I. Histology and Chemical Properties

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With two plates (figs. 5 and 6)

SUMMARY

Evidence is presented to show that the mesogloea of the coelenterates examined, including *Aequorea*, *Aurelia*, *Cyanea*, *Chrysaora*, *Calliactis*, and *Metridium* contains collagen-like material in the form of fibres of varying sizes. Cells are also present in the mesogloea of some species but there is little evidence concerning their functions.

In *Aequorea* and the Scyphozoa examined the fibres appear to be straight and very transparent in life but on injury and fixation they adopt a convoluted and irregular appearance. Cells are present in *Aurelia* and form about 0·3 per cent. of the mesogloea by volume but are absent from the other medusae examined. The mesogloea of actinians is of a more densely fibrous character than that of medusae. In *Calliactis* and *Metridium* the crossed fibrillar structure present is determined by mechanical forces acting on the tissue.

Reference is made to the physical properties of *Calliactis* mesogloea, which are described in another paper. The material is tough, almost resembling cartilage in its consistency, and has considerable tensile strength.

The chemical nature of the mesogloea is shown to be primarily that of a protein of which the amino-acid composition is similar to that of vertebrate collagen.

The role of the mesogloea in the life of the animal is that of a base for the muscles, a limiter of the body volume, and a resister of rapid movements, both those caused by the musculature and those enforced by the environment without. Its viscous-elastic properties are well adapted to this role since the elastic component, having a short time-scale, can provide a restoring force for rapid deformations.

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INTRODUCTION

OF connective tissues in general it is broadly true that they consist of a ground substance in which are present more or less independent cells and more or less independent and clearly defined fibres; this is true also of the coelenterate mesogloea. The ground substance of connective tissues does not generally show great affinity for stains, probably because its chemical constituents are of low concentration, but the fibres often stain strongly with basic dyes such as aniline blue. Although it is clear that the larger fibres are genuine structures it is often difficult to be certain whether or not the smaller fibres are artifacts, whether they are 'condensations' of or are separate from the matrix in which they lie and whether they are present in the living tissue or are a result of fixation. The possible origin of the fibres from the cells in the connective tissue is also a subject of controversy because the cells frequently appear to be so far away from the fibres as to make it appear unlikely that these were directly secreted by the cells. However, it seems generally to be accepted now that the fibres of connective tissue proper arise in the ground substance, outside the cell. For example, Baitsell (1925) concludes that in the chick embryo 'the fibrillation unquestionably arises by changes which occur in the ground substance', the forerunner of the connective tissue being the transparent, gelatinous, cell-free, ground-substance which pervades the embryo and which is 'evidently formed as a secretion of the cells of the various germ layers'. Harrison (1925) agrees that the fibres of the connective tissue of *Amblystoma* embryos are 'neither intra-cellular, nor are they formed in any particular outside layer which could be called exoplasmic unless the whole of the inter-cellular ground substance could be designated as such'. Wolbach (1933) concludes that collagen first appears in rats as a homogeneous material in which a reticulum of argyrophil fibres soon appears. Finally, Gersh and Catchpole (1949) believe the ground substance to be 'structurally organized, though on a submicroscopic or molecular level, micelles being bound to each other in such a way as to result in a medium whose consistency may vary'.

On whatever level the fibrous organization of connective tissues occurs it is clear that there must be, and that there is, a fibrous or long molecular orientated structure present. An attempt will here be made to show that an incipient or developed fibrous structure prevails which is in general accord with the mechanical properties of the connective tissue, with the structural requirements of the animal, and with the strains set up in the connective tissue by the movements of the animal.

Exactly how a condensation of ground substance comes about and produces the fibrous structure of the connective tissues of vertebrates is uncertain but it can be demonstrated *in vitro* that definite, fibrous, chemical compounds can be produced by mixing solutions of proteins with solutions of hexosamine sulphonic acids (Meyer, Palmer, and Smyth, 1937). It is thus conceivable that the conversion of a homogeneous proteinaceous matrix could be brought about by the secretion into it of a hexosamine sulphonic acid or similar carbohydrate by the cells of the tissues.

Whilst the protein collagen and amino-polysaccharides are of regular occurrence in vertebrate connective tissue it is, as might be expected, difficult to discern any common factor in the constitution of connective tissues of invertebrates. These animals possess skeletal materials with a variety of names—chitin, conchyolin, arthropodin, tunicin, and keratin—of varying chemical and histological nature. The supporting tissue of coelenterates is thus of particular interest from several points of view. The animals occupy a lowly position near the base of the metazoan stock so that they might be supposed to shed some light on the origin and composition of connective tissues in general. Secondly, in some coelenterates the bulk of the connective tissues relative to living matter is greater than it is elsewhere in the animal kingdom. Thirdly, there is a considerable range in the kinds of connective tissue found, from weak gelatinous to strong fibrous types. The fibrous nature of the mesogloea of various coelenterates has been recognized by many authors including the Hertwigs (1879), Mayer (1910), Stephenson (1928), Holmes (1950), and Russell (private communication), but none of these authors has sought to relate the fibrous structure of the mesogloea to its mechanical functions and properties, or to its chemical composition. Evidence will be presented to show that the bulk of the connective tissue protein material of the coelenterates examined conforms, in its histological appearance and physical and chemical properties, to the more clearly characterized collagen of vertebrates. This substance, of a fine fibrous texture, is strong and relatively inextensible, is associated with acid polysaccharides, contracts on heating, and stains with aniline blue and toluidine blue. Not every specimen of coelenterate mesogloea can be shown clearly to have all these properties but the sum of the indirect evidence to be presented makes it extremely likely that the basis of the connective tissues of coelenterates is collagenous in nature and is fibrous on a microscopic or a submicroscopic level.

In the first part of this paper an attempt is made to examine the histology of the mesogloea of species belonging to the three classes of coelenterates in order to see whether any common features can be discerned and whether they support the belief that the material is collagen. In the second part such chemical evidence as has been collected is presented in support of the collagen theory.

An account of the physical properties of the isolated mesogloea of *Calliactis* and *Metridium* is published elsewhere (Chapman, 1953).

HISTOLOGY

Materials and methods

All animals were used in as fresh a state as possible, the scyphozoans being caught at Whitstable and examined there or in London; the actinians were supplied by the Marine Biological Laboratory, Plymouth, and were examined there or in London. Observations were made wherever possible on living material which was examined with apochromatic objectives and with the phase contrast and polarizing microscope and was stained in some instances with neutral red or methylene blue.

A variety of fixatives was employed including 4 per cent. formaldehyde, Heidenhain's Susa, Bouin's and Helly's fluids, 90 per cent. alcohol, and the formalin-bichromate mixture of Tretyakoff (1937). Embedding was done in paraffin and the material cut into sections from 5 to 15 μ thick. These were stained in a variety of stains which included iron and Ehrlich's haematoxylins, Mallory's triple stain, Heidenhain's 'Azan' stain, Unna's orcein, Weigert's connective tissue stain, toluidine blue, and alcian blue 8GS.

Whilst some fixatives, such as Helly's fluid, are better than others, e.g. Bouin's, for the preservation of the fibrous structure of the mesogloea, it is true that fibres are present no matter how the material is fixed. Whilst it is true that the fibrous structure is sometimes difficult to make out in living tissue, it is usually possible to discern some trace of it and it is also possible to see that this remains during fixation. Hence there can be no doubt that fixed and stained preparations, while they may not be precisely the same in structure as the living material, present no gross misrepresentation of it. As will be seen below this agrees with the belief that the characteristic and regular arrangement of mesogloeal fibres is no chance effect of fixation. In the description given below of the organization of the fibrous structure of the mesogloea most attention has been given to sea anemones and to *Calliactis* in particular, but for ease of reference descriptions of the mesogloea of the various species has been arranged in the usual taxonomic order.

Aequorea vitrina

The jelly of this large hydromedusan, of 15 cm. diameter, is of a firm consistency, firmer than that of *Aurelia*, and is completely clear to the naked eye like that of *Cyanea* and *Chrysaora*. Microscopic examination of the living material in the unstained condition shows that it contains no cells of any sort but that the jelly is criss-crossed by very fine fibres. By watching fixation in formalin under the microscope it can be seen that the fibres which are visible in life are preserved in the fixed material. These are apparently most numerous near the surfaces of the animal, just below the ectoderm and especially on the exumbrella where they tend to run in the same plane as the surface. In places they have a spiral, or at least a convoluted appearance, but this may be due to the release of the natural tensions when the specimen is removed from the animal for examination. It is very probable that these spiral or convoluted fibres are due to the crumpling of a very fine, possibly submicroscopic, network of material in the jelly. By squashing the fresh jelly, fluid can be expressed and the fibres then become aggregated to form a tough tangle of material resembling areolar tissue. In material fixed in formalin and stained in Azan stain, a faintly coloured blue ground can be seen in which a 'condensation network' is apparent in places. This is most noticeable and stains most deeply immediately below the exumbrellar surface and between the cell layers of the manubrium. No cells are visible in the fixed mesogloea.

Aurelia aurita

During life the firm, slightly opalescent jelly which constitutes the bulk of *Aurelia* is so transparent that the cells are difficult to make out and the fibres more difficult still. Throughout the mesogloea the cells, as seen in sections, appear to be distributed at random; they make up only a small proportion of the bulk of the tissue. The ratio of the volume of living cellular material to matrix was determined as follows.

By using a one-sixth inch objective and counting the number of cells in a given area of a squared eyepiece for each $10\,\mu$ thick layer of the living tissue (as determined by focusing with a calibrated fine adjustment), it was found that in connective tissue taken from near the centre of the bell there are about $5{\cdot}6 \times 10^6$ cells per c.c. Each cell is approximately spherical and has a diameter of about $10\,\mu$ or 0·001 cm. so that in 1 c.c. there is approximately 0·003 c.c. of living matter, or about 0·3 per cent. by volume.

Examination of living material with all powers of the microscope failed to reveal any fine processes connecting the cells. Vital staining of the tissue with neutral red and with methylene blue enabled the mesogloea cells to be readily picked out. Large, heavily staining vacuoles were present in the cells, but other cell matter did not appear to take up the stain. These are presumably the 'neutral red vacuoles' of the Golgi apparatus since it is not the nucleus which stains selectively with neutral red. It appears that the same vacuoles are picked out by methylene blue used as a vital stain. In the living material the nucleus can just be made out as a large body slightly more refractive than the cytoplasm.

The use of the phase-contrast microscope on hand sections of the living mesogloea of young jellyfishes (up to 10 cm. in diameter) failed to show any fibrous structure other than slight traces of straight fibres. The typical network obtained in sections of fixed material could not be seen, nor could Tretyakoff's spiral fibres; so it may be that the network is due partly, if not entirely, to fixation and dehydration. Likewise the cells of the mesogloea revealed nothing under phase-contrast observation that cannot be seen by the use of ordinary light and an apochromatic objective. No interconnecting strands between the cells could be made out.

In the present investigation of *Aurelia*, the material was fixed in the formalin and dichromate mixture of Tretyakoff (1937) as well as in other fixatives and was stained in Ehrlich's haematoxylin, Mallory's stain, Azan stain, and toluidine blue. No matter what fixative was used the cells could be clearly seen and presented an appearance not dissimilar to those seen in the living material. They were somewhat smaller than the living cells, being about $7\,\mu$–$8\,\mu$ in diameter. The 'neutral red vacuoles' did not appear in the fixed and stained material. These vacuoles are stained by toluidine blue *in vivo* but after fixation this stain readily dyes the whole cell and also the fibres, both of which take on the purplish tint of the metachromatic form said to be characteristic of mucoproteins (Lison, 1936).

It seems unlikely on such histological evidence (which is corroborated by the absence of cells from other species of Scyphozoa and from some species o Actiniaria, all of which have a well developed mesogloea) that the cells of th mesogloea secrete either the fibres or the matrix, the origin of both of which i unknown (Hyman, 1940). In *Aurelia* the cells of the mesogloea do not posses the characters of wandering cells or amoebocytes and it is not easy to believ that they have wandered in from the ectoderm because there does not appea to be any particular concentration of cells near the ectoderm as if they wer migrating from there; nor do they appear amoeboid in life. As is mentione

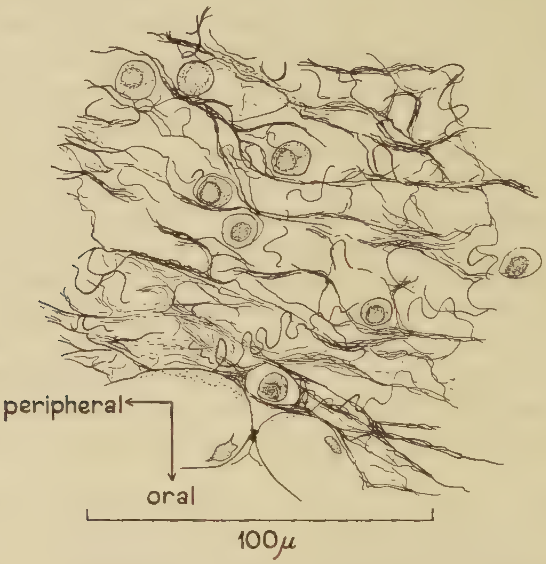

FIG. 1. Vertical radial section of mesogloea of *Aurelia*, stained with toluidine blue.

elsewhere (pp. 158 and 161), in some other jellyfishes no cells at all have been seen in the mesogloea, so that they could not have been the agency for the secretion of the fibres or of the matrix.

The fibrous nature of the fixed material is clear no matter what stains are used. With the polychrome stains such as Azan and Mallory's the network is blue, which suggests its affinity with the collagen of vertebrates. The fibres also stain well with toluidine blue and in such preparations fibres of all sizes can be seen. Some of the larger ones have a spiral or at least a convoluted appearance but most are of extremely small size and appear, in the words of Stephenson (1928), to be 'condensations of the matrix'. It is extremely difficult, even with an apochromatic one-twelfth inch oil immersion objective, to be certain that the finest fibrils have a structure or an identity which is separate from the general matrix of the mesogloea. The larger fibres appear to be aggregations of the smaller ones in which the component fibrils are discernible with varying degrees of clarity. The appearance of a toluidine blue preparation is shown in fig. 1.

There appears to be no difference between the staining properties of the smaller and the larger, or spiral, fibres and they cannot always be clearly distinguished from the reticulum. It is not certain that the fibrils forming the more finely reticulated structure are present in life as the same structural entities that can be seen in stained preparations. The finer fibres cannot be seen during life even under the phase-contrast microscope, although the larger fibres can be seen, usually as straight but sometimes as convoluted elements. These fibres can be seen to persist during fixation with formalin and with alcohol and there is no doubt that the larger fibrous elements seen in fixed material are identical with those seen in the living tissue. That the finest fibres, and such traces of matrix as appear coloured, possess the same staining properties as the larger ones suggests that they are of the same chemical nature (i.e. probably collagenous), but whether they are present as organized fibres in life is uncertain. It may well be that there is a submicroscopic structure in the jelly, similar to that of the larger fibres but on a smaller scale and made of the same material.

Other animal jellies have a similar appearance. For example, stained sections of the egg-cocoon of the polychaete worm *Scoloplos armiger* have a finely reticulated structure although the fresh jelly seems almost homogeneous, the merest trace of fibres being visible under the phase-contrast microscope.

Cyanea lamarcki

The connective tissue of *Cyanea lamarcki* is more transparent during life than that of *Aurelia* and when separated from the cellular layers is practically invisible in sea water. Examination in the fresh condition failed to reveal the presence of any cells, nor were any found in sections fixed in formalin and dichromate and stained in Azan or toluidine blue. Neutral red staining of the living tissue also failed to show the presence of any cells although the ectodermal cells took up the stain very easily.

The fibrillar structure is less easy to discern than that of *Aurelia* but in the fresh condition very fine criss-cross straight fibres can be made out.

The appearance of the tissue when fixed and stained is similar to that of *Aurelia* except that the concentration of fibres is very much less and the spiral fibres appear to be lacking. There is some variation in fibre size and the same difficulty in distinguishing the finest fibres from the ground substance. (See fig. 2.)

Chrysaora mediterranea

The mesogloea of *Chrysaora mediterranea* is transparent in the living condition and contains no cells but has a noticeable fibrous structure. By examination of thick, hand-cut, radial sections it could be seen that the exumbrella had a better developed fibrous structure than elsewhere. Fibres could be seen running in all directions parallel with the surface. Also, running at right angles to the surface and ending in the superficial network there are large fibres or fibre aggregations which have a branching arrangement at each end where they approach the surface (fig. 3). These fibres cannot easily be seen in sections of material cut at 5 μ but this may be merely because there are few fibres and the sections examined had not chanced to pass along one. On the other hand, the superficial layer shows clearly as a more deeply staining layer in sections of fixed material. Individual fibres are hard to discern although the layer has what may be called a fibrous appearance. The amount of material that can be stained is small and the fibrous structure of the bulk of the mesogloea which lies between the exumbrella and the subumbrella is very poorly developed.

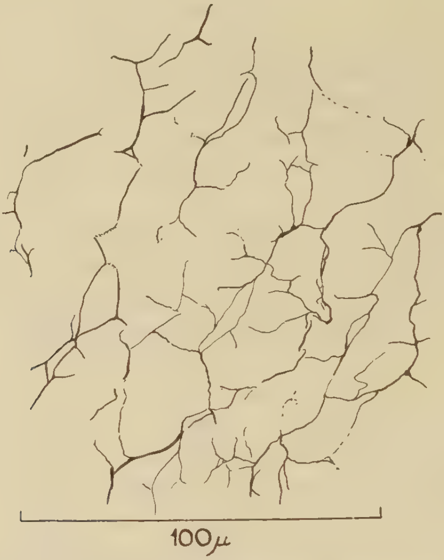

FIG. 2. Vertical radial section of mesogloea of *Cyanea*, stained with toluidine blue. Note absence of cells and sparsity of fibres.

Calliactis parasitica

The mesogloea of *Calliactis parasitica* is a thick fibrous layer which, when fixed, stains blue with Azan and with Mallory's stain and red with van Gieson's connective tissue stain. As in the jellyfishes there are fibre aggregations of various sizes between which there is an apparently structureless matrix whose content of stainable material is very small indeed. In addition there are cells, which appear to be all of one type, and which are present throughout the mesogloea but which occur in greater concentration near the endoderm than elsewhere. Whilst the demonstration of the cells and fibres in stained sections is straightforward, attempts were made to find if there were any other components which might have been expected to be more easily demonstrable by other means.

Hand sections of the freshly excised material were stained with neutral red and with reduced methylene blue, but even the cellular elements did not stain as successfully as they did in the Scyphozoa. Sections of fixed material were stained in Unna's orcein, Weigert's elastin stain, mucicarmine and toluidine blue, but no trace of other types of connective tissue fibre could be demonstrated and the toluidine blue did not appear to give the purplish colour with the fibres or the matrix which it yields with those of the Scyphozoa tested. It was not possible to demonstrate any fat in fresh sections by the use of Sudan III.

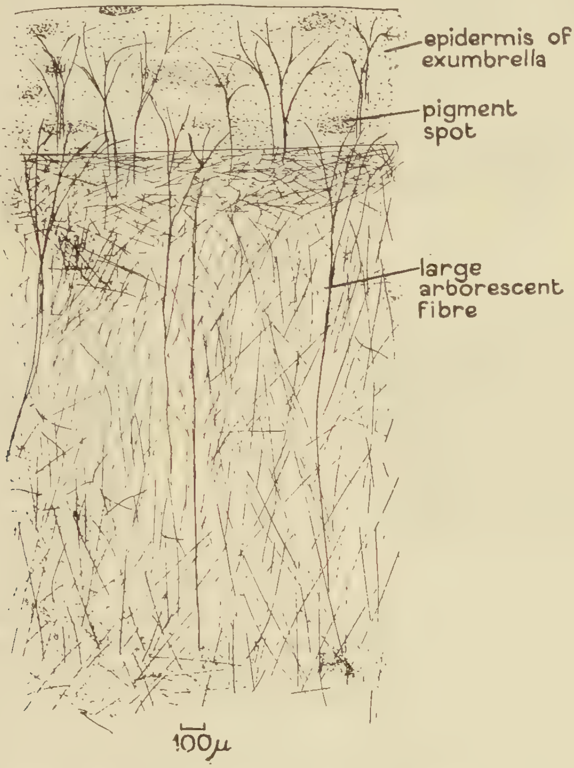

FIG. 3. Vertical thick section of exumbrellar region of *Chrysaora*, during life. Unstained. The ectoderm is represented by stippling and the ends of some of the larger arborescent fibres are seen through it.

The fibres of which the mesogloea is composed vary in size from near the limit of microscopic resolution to aggregations of 5 μ in diameter. They appear to anastomose freely, to run for a while parallel with their fellows and to branch off so that the appearance of a microscopic section is at first sight one of a tangled structure. While they can nowhere be said to run all in one direction they can be seen to have clearly defined predominating directions which have a characteristic pattern. In general terms it can be said that their distribution appears less regular in the middle of the mesogloea and that orientation appears increasingly well ordered towards the inner and outer sides. This pattern can be made out by cutting sections in a transverse, radial and tangential manner and at 45 degrees to the long axis. Diagrams illustrating the fibre arrangement as seen in sections cut in this way are shown in fig. 4. The characteristic patterns are more or less clearly seen in all sections but the amount of ordered arrangement shown by the fibres is perhaps more clearly seen in well expanded animals than in contracted ones.

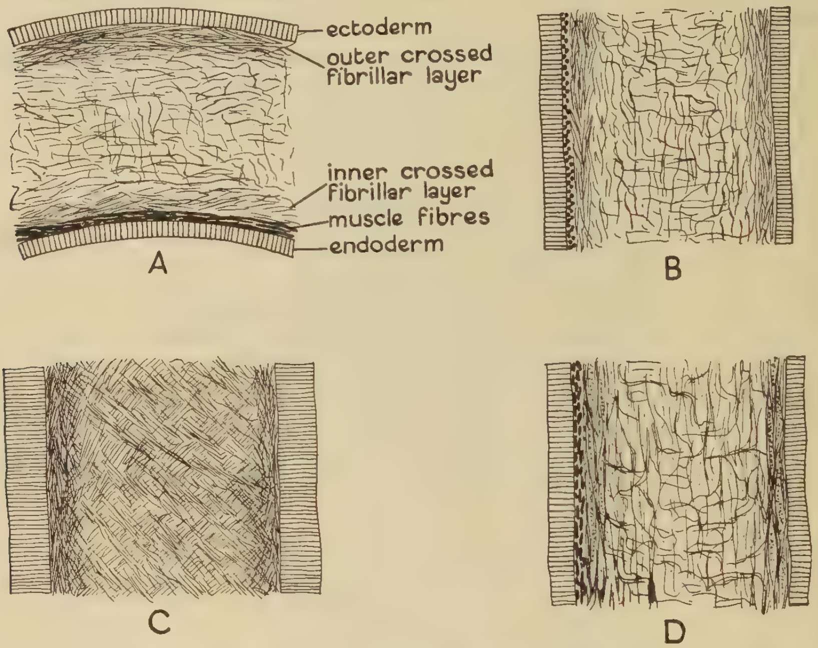

FIG. 4. Diagrams illustrating the general appearance of the body-wall of *Calliactis* as seen in (A) horizontal section, (B) radial section, (C) tangential section, and (D) section at 45 degrees to the long axis. Note in A and B the similar appearance of the inner and the outer crossed fibrillar layers and the middle, less highly orientated region. Note in C the outer crossed fibrillar layer showing warp and weft arrangement, and in D the crossed fibrillar arrangement in which the fibres are cut either along their length or in transverse section, and are represented by dots. The interconnexions of the sheets of fibres are shown.

These fibres are arranged not only in sheets parallel with the surface and with the fibres at 45 degrees to the long axis of the animal but also with some fibres traversing the thickness of the mesogloea and running from one sheet of the crossed fibrils to another. In the inner and outer layers of the mesogloea the fibres are arranged predominantly parallel with the surface so that a tangential section presents the appearance of a warp and weft arranged, as it

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FIG. 5 (plate). A, tangential section of mesogloea of *Calliactis* near the ectoderm, showing the crossed fibrillar structure. Oral-aboral axis vertical. Mallory's triple stain. B, section of mesogloea of *Calliactis* at 45 degrees to long axis, showing that the crossed fibrillar arrangement of sheets of fibres is more pronounced towards the outside of the mesogloea (left) and less towards the centre (right). Sections of fibres cut across (appearing as dots) can be seen to alternate with fibres running in the plane of the section (45 degrees to long axis) more noticeably on the left than on the right, where, in the centre of the mesogloea, the forces orientating them are less effective. Mallory's triple stain.

were, diagonally to the long axis (figs. 4, C and 5). However, in such a tangential section the fibres are nowhere cut for any great distance along their length because they do not run exactly parallel with the surface, but, as in a woven fabric, have the form of undulating sheets interlocked with those that run at right angles to them. The fibres thus form a lattice structure, not only in two dimensions parallel to the surface but also in the third dimension at right angles to it.

It is likely that this arrangement of fibres arises from the action of mechanical forces on the mesogloea in similar manner to the arrangement in *Donacia* cocoons described by Picken, Pryor, and Swann (1947). They showed that when the cocoon of the beetle is first deposited round the larva it is an amorphous viscid mass which afterwards hardens and becomes laminated and can be shown to be composed of chitin chains arranged at 45 and 90 degrees to the long axis of the barrel-shaped structure. They argue from the example of *Donacia*, and from other observations, that the orientation of long molecular chains in preferred directions is probably not a result of an orientating process of the cells which produce the structure but rather a result of the mechanical forces acting on it during its deposition. In like manner it will be seen that the fibrous structure of the mesogloea of *Calliactis* agrees with its production from a random arrangement of developing fibrils into which order is brought by the stresses to which it is subjected. It is to be expected that any extension or compression of the cylindrical structure will produce strains in the wall, causing the fibres to become orientated in a 'crossed fibrillar' pattern which is the best arrangement of skeletal material in such an object undergoing changes of shape at constant volume. The way in which axial compression can cause the appearance of diagonal lines of strain in a plastic material is shown in fig. 6. The photograph shows a cylinder of cast phenol-formaldehyde resin after a compression test: two sets of diagonal cracks can be clearly seen. The specimen was kindly lent to me by Dr. M. G. M. Pryor. Similarly, stresses are set up in the mesogloea of *Calliactis* by the muscle system which lies on its inner side. Both the circular muscles, which act directly, and the longitudinal muscles, which act through the agency of the coelenteric fluid, are situated on the inner side of the mesogloea; so it is to be expected that the inner side will show the crossed fibrillar structure most clearly and to the greatest extent.

That the arrangement of the fibres is due to the orientating effect of mechanical forces implies that there is no differentiation or boundary between the layers of the mesogloea which have been mentioned: and it can be seen, in the region where the outer regular crossed fibrillar orientation is giving place to the less conspicuously diagonal orientation of the centre, that the fibres are

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FIG. 6 (plate). Cylinder of phenol-formaldehyde resin (kindly lent to me by Dr. M. G. M. Pryor) after a compression test, showing crossed diagonal lines of strain in the surface layer. This illustrates the forces which may operate also on the fibres of *Calliactis* mesogloea. Those not conforming to the pattern would be broken during the changes of shape brought about by muscular action. (Photograph by Mr. Jack Skeel.)

continuous and that those of the centre become, as it were, incorporated in the more conspicuously regular pattern as they approach the outside layer. This can be seen in fig. 5, B.

A living anemone undergoes great changes of shape and volume. The crossed fibrillar or geodetic method of construction clearly allows the changes of shape to take place easily under the influence of the circular and longitudinal muscles acting with the hydrostatic skeleton. Changes in *shape* over the ordinary living range produce little change in thickness of the mesogloea and a two-dimensional crossed fibrillar construction alone would have to undergo very little crumpling or stretching on its fibres. However, when changes in *volume* of the animal occur, changes in thickness of the mesogloea are involved and hence a fibrillar structure in two dimensions only would not suffice unless the layers of fibrils were radially separable when contraction of the animal and thickening of the mesogloea occurs. That *Calliactis* approximates to this in its construction is shown best by the examination of sections of expanded and contracted animals at 45 degrees to the long axis. In those from the expanded animal the mesogloea is thinner and the lamination of the inner and outer regions of the mesogloea is greater. In those from the contracted animal the mesogloea is thicker and the crossed fibrillar laminations less in evidence because more fibres are running from one tangential layer to another and are also inclined at a steeper angle to the surface than they are in the thinner mesogloea from the expanded animal. While there is still some appearance of crossed fibrillar arrangement in the middle of the mesogloea of the contracted animal, on account of the greater separation of the fibre layers and of their interlocking fibrils, this is more difficult to see and it is possible sometimes to observe an almost radial fibre arrangement resulting from the increased thickness of the mesogloea pulling the fibres into alignment across the thickness of the mesogloea.

Although the way in which the mesogloea arises from the cellular layers is not clear, there is little doubt that the fibres which make up its most important structural part are orientated by the forces of the muscles and of the environment which are exerted on the anemone during its life and that they do not owe their particular arrangement to the agency of any organized method of secretion by individual cells. It is hoped that this general principle will be illustrated by more examples drawn from the coelenterates when more species are examined in greater detail than has been possible for the preparation of this paper.

The cellular elements of the column mesogloea, which are variable in their number and distribution, are most easily seen in tangential section and are most numerous in the inner diagonal fibrous layer. The cells are irregularly shaped and their appearance varies from one preparation to another. In Azan preparations they are seen to contain numerous granules which stain red, as a consequence of which they stand out very clearly from the mesh-work of blue-stained fibres in which they are embedded. From their appearance in transverse, radial, and tangential section the processes from the cells appear to be

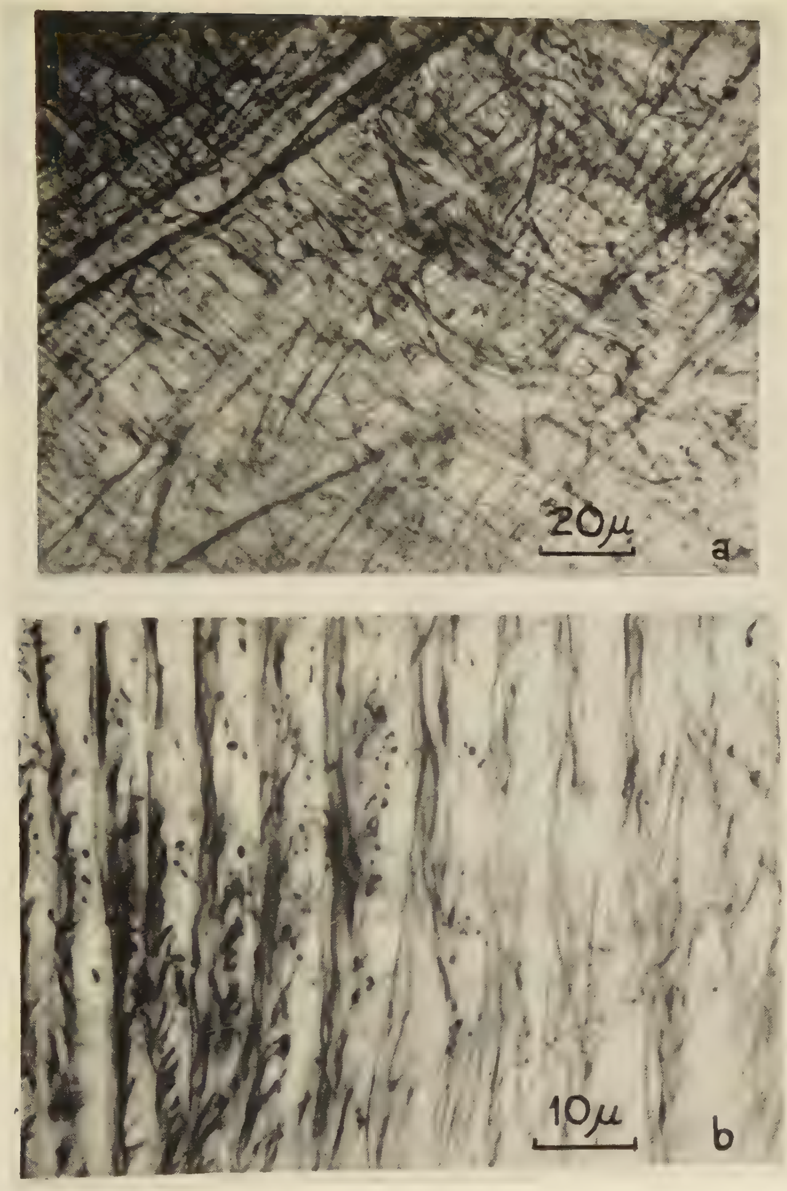

Fig. 5
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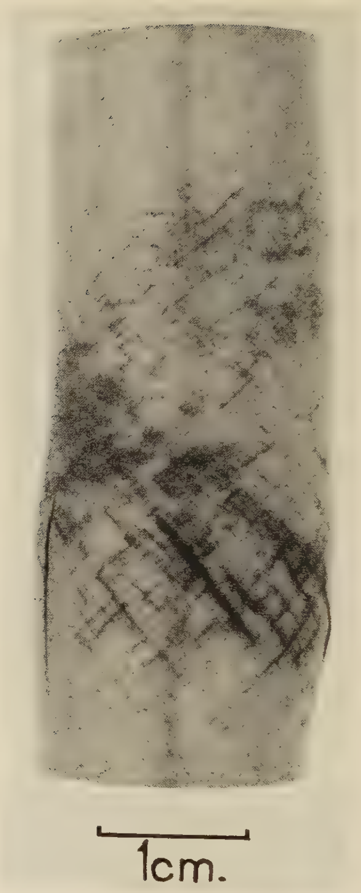

FIG. 6
G. CHAPMAN

spread out mainly in a plane parallel to the surface of the animal. As with the cells in the mesogloea of jellyfishes they are unlike secretory cells in appearance and the granules which they contain do not have the same staining reactions as the fibrous portions of the mesogloea. They are predominant near the circular muscle layer and although in tangential section they may often be seen lying alongside the muscle fibres they do not appear to be connected with them. Although they bear a superficial resemblance to neurons, they are almost certainly not nervous elements because these have been demonstrated in *Metridium* by Batham and Pantin (1951). The undoubted neurones which they demonstrated are fewer in number than the mesogloea cells, stain with reduced methylene blue and with silver, and possess long axons which clearly form an interconnected system. The mesogloea cells resemble fibroblasts and were described (as connective tissue cells) by O. and R. Hertwig (1879*a*).

Other actinians

The general appearance of the mesogloea of *Metridium senile* as seen in section is very similar to that of *Calliactis* except that it is a considerably thinner layer in the expanded animal and the division into three layers is very much less clearly shown, as would be expected in an animal with a thinner mesogloea.

In *Edwardsia callimorpha* the fixed mesogloea appears to be devoid of cells but to have a well developed fibrous structure which is thickest near the strong endodermal muscles. The nemathybomes (hollow sacs containing large nematocysts) interrupt the mesogloea at intervals.

Bolocera is sometimes spoken of as having a 'chondroid' type of mesogloea, but in *B. tuediae* at least the mesogloea is fibrous. This sea anemone is large and has a thick mesogloea of which the inner portion is abundantly fibrous and resembles that of *Calliactis*, although *Bolocera* has, perhaps, fewer cells. In tangential section the same warp and weft structure is seen as occurs in *Calliactis*, especially in the thick inner fibrous layer.

Discussion

The impression left by this survey of the connective tissues of eight different species of coelenterates is the general resemblance of the fibre-matrix element in all the types examined. The differences between them appear to be differences of degree rather than of kind. In one species the fibre-matrix is watery and with few fibres, in another the fibrous structure is well shown. Nowhere, however, does the fibre structure look like the separable, clearly defined fibrous structure seen in some vertebrate collagenous tissues and indeed the evidence from living and fixed material and from the physical properties of the tissue (Chapman, 1953) points to the very imperfect separation and distinction of the fibres from the ground substance during life. The cellular components of the mesogloea are variable and may be absent altogether, which hardly suggests that it is the mesogloea cells which secrete the mesogloea; indeed no evidence is forthcoming to account for its origin.

In the Hydrozoa great enlargement of the mesogloea rarely occurs, so that *Aequorea* is an unusually favourable member of the class for study. Whilst no evidence directly supporting his conclusions has been obtained in the present work, Holmes (1950) has suggested that the muscle fibres of *Chlorohydra* are in reality 'specializations of the mesogloea' which, he thinks, is fluid; and, indeed, it has been shown by Harris, Sloane, and King (1950), using freeze-drying techniques, that the mesogloea of *Hydra* is thicker than it is normally seen to be in fixed material.

Aurelia is different from the other Scyphozoa examined in the course of the present work in having cells in the mesogloea. They are not amoeboid, as Tretyakoff (1937) agrees and do not, as far as can be seen, possess the fine connecting processes described by Schultze (1856). It was not possible to distinguish in the Scyphozoa the great variety of fibres and fibrils described by Tretyakoff.

Among the actinians the mesogloea is far more densely packed with fibres and fibrils than it is in the Scyphozoa but it is of similar histological appearance. The fibrous structure is clearly visible in the unstained living material and stains densely with aniline blue when fixed. As with the Scyphozoa, some species possess cells in the mesogloea while others do not. The cells therefore cannot be essential for the secretion of the fibres and in this class, as among the Scyphozoa, the cells do not appear to be in any way connected with the fibres.

The arrangement of the fibres in the column wall of *Calliactis* and other sea anemones has already been discussed on a functional basis.

CHEMICAL PROPERTIES

Introduction

Whilst there is practically no information about the chemical composition of the Hydrozoa or of the Actiniaria, there have been several published analyses of Scyphozoa. For example, Krukenberg (1880) states that the mesogloea of *Aequorea* and *Rhizostoma* yields neither gelatin nor mucin and is thus chemically unrelated to connective tissue and that the entire medusae of *Rhizostoma*, *Aurelia*, and *Chrysaora* contain about 95–97 per cent. water. According to Hatai (1917) the water-content of *Cassiopeia* jelly is 94·6 per cent., and of the cellular parts is 93·8 per cent. He was not of Krukenberg's opinion about the chemical affinities of the jelly and suggested that it might be related to cartilage, mucin, and chitin. According to Lowndes (1942) the water-content of *Aurelia* of 4 in. diameter is 95·56 per cent., but Thill (1937) states that it may rise to 98 per cent. in brackish water. Since jellyfishes are in approximate osmotic equilibrium with the sea water which contains about 3 per cent. of salts (Robertson, 1939) and since the creatures contain about 95–98 per cent. water, their content of solids other than salts must be extremely small and probably does not greatly exceed 1 per cent. and may be less.

The chemistry of vertebrate connective tissue has recently been studied intensively and the main features of its chemical architecture are becoming clear. Briefly it has been shown that the chemistry of skeletal proteins and mucoproteins is closely linked and that the two are usually closely associated even in such functionally diverse structures as cartilage and tendon. In general the protein is of the insoluble type, collagen, while the mucoprotein is of a variety of types and is easily split off by chemical, and even by physical, agencies from the protein with which it is associated: moreover, the carbohydrate part of the molecule may be easily separated from the protein part of the mucoprotein itself. The precise relation between collagen and mucoprotein does not seem to be clearly defined, although evidence for their association is considerable. For example, according to Partridge (1948*a*) the conditions which bring about a good yield of mucoid from beef nasal septum are the same as those which give rise to the termal contraction of collagen. He found that it was not possible to liberate mucoid from nasal septum without modifying the collagen, so that it may well be that mucoid, as such, is not present in the original tissue. He says that 'if our knowledge of the chemical behaviour of the polysaccharides is combined with the results of histological investigations the structure may be visualized as a network of collagen fibrils, in some places organized into parallel bundles to form microscopic fibres and in others relatively disorganized and heavily cross-linked by association with chondroitin sulphate. The contraction experiments also suggest that the protein of the mucoid springs from the disordered collagen of the cementing substance rather than from the ordered fibres.' Bradfield (1950) in discussing collagen and reticulin suggests that, where plasticity is required in connective tissues, as in the embryo, 'the connective tissue fibres are leavened with a pinch of polysaccharide'.

Evidence from X-ray diffraction studies also points to the similarity between the collagens and the mesogloeal fibres of *Aurelia* (Astbury, 1940) and of various Alcyonaria (Marks, Bear, and Blake, 1949).

Material

After excision of a piece of body-wall from living *Calliactis* all coloured ectoderm was cut off and the inside scraped to remove endoderm, after which the pieces of mesogloea were preserved in 70 per cent. ethanol. The gonads, oral arms, and the edge of the disk were removed from *Aurelia* before the remains of the animals were pickled in 70 per cent. ethanol. On some occasions ethanol was not available and 4 per cent. formaldehyde was used instead. After fixation the jelly was dialysed in a cellophane bag against tap water until the surrounding liquid gave only a faint indication of chloride when tested with acid silver nitrate. The dialysed jelly was air dried at room temperature and the dry residue scraped off the enamel pie dishes in which the concentration had taken place. The jelly of *Cyanea* is so transparent and so large a specimen was obtained (40-45 cm. in diameter) that only parts of the mesogloea were kept in which it could be seen that no cellular layer was present. (It will be recalled that there are no cells present in the mesogloea of *Cyanea lamarcki*.) No alcohol was available, so the material was fixed in formalin and treated subsequently as was *Aurelia*. *Chrysaora* was preserved, cell-free, in alcohol and was dialysed as usual.

For comparison of the amino-acid content, rat tail tendons from alcohol-fixed stock material and commercial blood albumen were used.

Methods and results

In preliminary tests it was observed that both *Calliactis* and the dialysed, salt-free, scyphozoan residues retained powers of absorbing water when prepared as described. Strips of *Calliactis*, dry for 2 months, very soon gained what appeared to be their normal size when immersed in water: after 2½ years in store they still swelled considerably when soaked in water. The jelly-like consistency of the evaporated *Cyanea* and *Aurelia* could also be partially restored by water.

The results of other preliminary tests which were made on the fresh mesogloea of *Calliactis* are listed below.

(i) The material was insoluble in boiling water but was rendered soft and translucent.
(ii) The material was soluble in boiling N. hydrochloric acid.
(iii) When material was dissolved in boiling N. hydrochloric acid and was brought to neutrality (B.D.H. Universal indicator) the solution gave a positive indication with the biuret test.
(iv) The xanthoproteic test was applied to fresh intact mesogloea with a positive result.
(v) The ninhydrin test was applied to the neutral solution prepared as in test (iii). A positive result was obtained.
(vi) The arginine test was applied to the neutral solution and a positive result was obtained.
(vii) Molisch's test for sugars was applied to the neutral solution, but no sugar could be detected with certainty.
(viii) The rapid furfural test for sugars was applied to the neutral solution, but again no sugar could be detected with certainty.

There is thus no doubt that the material is proteinaceous but it remains uncertain whether any carbohydrate is present.

A few tests were made on the mesogloea of *Chrysaora* which, like that of *Cyanea*, can easily be prepared cell-free. A few pieces were boiled in tap water, when they shrank to a very small fraction of their original size. The water and the tissue were boiled with ninhydrin solution and the tissue became dark purple and the ninhydrin solution very slightly blue. This test suggests that the protein in the mesogloea is largely in the form of material which contracts on heating; this again is consonant with the behaviour of collagen fibres. The material between the fibres would appear to contain very little protein indeed. The boiling experiment was repeated quantitatively in an attempt to gain some idea of the fraction of the wet weight of the animal jelly that could be regarded as protein.

About 77 g. of cell-free mesogloea of *Chrysaora* was boiled in a small quantity of tap water for a few minutes until it appeared to shrink no further. The remains were picked out by hand, placed in a watch-glass and dried in a desiccator until of constant weight. The water in which the material had been boiled was evaporated to dryness and the weight of the residue, largely salt, was determined. The results showed that, of the wet weight of *Chrysaora*, 3·4 per cent. is soluble in boiling water and, clearly, is largely salt, whilst only 0·11 per cent. is insoluble and is largely proteinaceous in character. It is probable that this figure of 0·11 per cent. is somewhat too high, because even the contracted protein had some water adhering, which contained salt. Also it is difficult to remove the last traces of water from proteins, which normally contain a residual 10–15 per cent. of water.

Amino-acid composition of mesogloea proteins

An examination of the amino-acids making up the protein portion of the mesogloea of various coelenterates was made by the method of paper partition chromatography. It was hoped that this would show a considerable similarity between the amino-acid composition of the coelenterate proteins and that of a typical collagen and that differences could be demonstrated between the coelenterate and other proteins not belonging to the collagen group, e.g. blood albumen. Although similar qualitative amino-acid composition cannot be regarded as proof of protein similarity in other particulars it does, nevertheless, provide additional indirect evidence for resemblance between groups of proteins.

To prepare the material for test it was hydrolysed under reflux for not less than 16 hours with 6N. hydrochloric acid, evaporated to dryness and redissolved in distilled water three times and finally filtered. It was kept, before test, in a refrigerator and was covered with a layer of benzene.

The method of paper partition chromatography which was found satisfactory was the use of the two-dimensional technique, the paper being 'run' first in one direction with a methyl cellosolve mixture and then being 'run' in a direction at right angles to the first with a butanol mixture. The composition of the first solvent was: methyl cellosolve 4 vols.; butanol 1 vol.; 0·5N. ammonium hydroxide 2 vols. (R. W. R. Baker, private communication). The second solvent was prepared by shaking together 5 vols. of butanol, 1 vol. of glacial acetic acid, and 4 vols. of water, and taking the top fraction (Partridge, 1948). A beaker containing some of the bottom, aqueous fraction was placed in the tank in which the 'runs' were made to ensure constancy of composition of the solvent mixture.

A spot containing 10 or 20 μl. of the hydrolysate was placed 3 in. from the left and from the bottom edge of a 15 in. square of Whatman 3 MM filter paper. After drying the spot the paper was rolled into a cylinder, clipped, and placed erect in about 40 ml. of solvent in a 6 in. Petri dish in an enclosed glass tank. This, and another similar tank, was enclosed in a large box which was thermally insulated but which had no temperature control. When the solvent front had reached to within 3 in. of the upper edge of the paper this was removed from the tank, dried, unrolled and rolled again for the 'run' in the second solvent at right angles to the first. After drying the paper again the centre 9 in. square was sprayed with a 0·1 per cent. solution of ninhydrin in butanol and was developed either by careful heating in front of an electric fire or by being left at room temperature overnight. The latter method has the advantage that the colour subsequently fades less quickly. For the identification of amino-acids in the hydrolysate 'runs' on separate papers were made with known amino-acids.

The appearance of the patterns provided by the hydrolysates of the mesogloea of *Calliactis*, *Aurelia*, *Chrysaora*, and *Cyanea* and by that of rat-tail tendon were closely similar, both in the amino-acids present and in the relative intensities of the colours which they gave with ninhydrin. Some idea of the similarities and differences can be gained from Table 1, which gives a rough idea of the relative amounts of the nine regularly occurring amino-acids which were present in the six hydrolysates examined. Although, in general, the same amino-acids were present in the albumen hydrolysate as were present in the collagenous materials their proportions were somewhat different and there were three clearly defined spots belonging to additional amino-acids which were not identified but which were not present in the collagens. There was also only a rather doubtful trace of proline in the albumen, although it, and often hydroxyproline too, were present in the collagens.

TABLE 1

Amino-acid composition of coelenterate collagens

	Calliactis	*Aurelia*	*Cyanea*	*Chrysaora*	*Rat tendon*	*Blood albumen*
Alanine	++++	+++	++++	+++	+++	+++
Glutamic acid	++++	++	++	+++	++	++++
Glycine	++++	+++	+++	++++	++++	++++
Histidine	++	+	? +	? +	?	+++
Leucine	+++	+++	+++	+++	+++	+++
Lysine	++	++	+	++	++	++
Proline and hydroxyproline	++	++	++	+++	+++	?
Serine	+	+	? +	+	?	++
Valine	++	+++	+++	++	++++	++
						3 additional amino-acids

Whilst it can hardly be claimed that the differentiation between the various proteins examined is striking, it can be stated that the amino-acid patterns revealed by the method of paper partition chromatography show a great similarity in the collagen–mesogloea group and that this is distinguishable on examination from another protein such as blood albumen. This again provides evidence that the protein material found in the mesogloea of coelenterates is of a collagenous nature.

THE ROLE OF THE MESOGLOEA

In the section on histology it was seen that in some representative coelenterates the mesogloea is a fibrous structure in which the fibres are too small to be distinguished as clearly separate from the tenuous matrix in which they lie and of which they appear to be condensations. It cannot be doubted that this fibrous structure plays a part in the life of the animal, but the importance of the cells which occur in some species is not clearly understood because there are other closely related species from which they seem to be absent. It has been suggested (Pantin, private communication) that they are present in those coelenterates which can absorb the mesogloea during starvation, but that they are absent in those which cannot. No direct mechanical measurements have been made on Scyphozoa, but the experiments which have been made on *Calliactis* (Chapman, 1953) have shown that, despite its fibrous structure, strips of mesogloea cut in different directions have little difference in tensile strength. This is probably due to the approximate alignment of the fibres with the direction of stress. The material as a whole behaves in a viscous-elastic manner which is also in accord with the evidence that, chemically, the mesogloea can be regarded as a collagen-like protein associated with little, if any, polysaccharide and probably much less highly organized than the micellar protein of vertebrate connective tissue fibres.

There can be little doubt that the role which the mesogloea plays in the life of the animal is primarily an inert mechanical one, although it has been postulated (Holmes, 1950) that argyrophil fibres in the mesogloea of *Chlorohydra* are contractile and are the 'muscle tails' of previous authors. No evidence has been obtained in the present work to support an extension of this view to include other coelenterates. The excised mesogloea of *Calliactis*, even after prolonged anaesthesia, does diminish in volume on handling and liquid exudes from it but there is no evidence for the existence of a contractile process in the mesogloea itself.

It is likely that the stability of the whole mesogloea may depend on its preservation entire or as an organized system and that a cut may release elastic tensions which, in life, are balanced by others. The preservation of the shape of a jellyfish appears to depend on the preservation intact of its fibrous structure; fibres which are straight in life may be twisted when their anchorage is cut or after fixation. Thus it is likely that the fibrous skeleton is normally kept taut and distended by a slight hypertonicity of the intercellular matrix due to electrolyte or to colloid osmotic pressure. There does not appear to be any mechanism in the Scyphozoa analogous to the rosette openings which, in ctenophores, put the mesogloea into communication with the enteron and which may serve to control the turgor of the mesogloea.

The excised, compacted mesogloea of *Calliactis* is of a cartilaginous consistency and there is little doubt that in life it contributes to the maintenance of the shape of the animal. There are, however, other anemones in which the mesogloea is too weak to support even the weight of the animal in water. Batham and Pantin (1950) have shown that a fully extended *Metridium* may topple over by its own, very small, weight in water. It has also been shown (Chapman, 1949) that *Calliactis* works chiefly as a closed, fluid-filled, muscular sac in which one set of muscles antagonizes the others by virtue of the contained fluid, and that *Metridium* (Batham and Pantin, 1950) works partly in this way and partly as an open sac which is refilled, after emptying, by the ciliary action of the siphonoglyphs. These produce pressures which are small but which are just adequate to inflate the wrinkled body-wall. Such observations, as well as those made on excised material (Chapman, 1953), demonstrate the power of small forces to deform the mesogloea when they act for a sufficiently long time. On the other hand, there is little doubt that the mesogloea can play a skeletal or supporting part and is important to the animal in resistance to imposed forces, especially those of short duration.

The mesogloea has considerable tensile strength (Chapman, 1953) and may play a part in setting a limit to the volume which the animal can attain or at least, by virtue of its viscous-elastic properties, to the rate at which it can increase in volume. The mesogloea can also be regarded as an antagonist to muscles such as the mesenteric retractors which, in *Metridium* (Batham and Pantin, 1950) can set up pressures in the coelenteron of 10 cm. of water while their muscular opponents, the circular muscles of the column, can withstand a coelenteric pressure of only 1·0 cm. of water. In an animal of 5 cm. diameter a pressure of 10 cm. of water corresponds to a circumferential tension of 25 g./cm. (Chapman, 1950). If the mesogloea were of 50μ thickness the tension in it might rise to 50 g./sq. mm., which is very much less than its tensile strength of 250–400 g./sq. mm. The mesogloea, even if it were only 10μ thick, would therefore be well able to withstand the stress set up by 10 cm. pressure in the coelenteron. If this pressure were set up by a rapid contraction of the mesenteric retractors, the stress set up in the body-wall would be borne by the mesogloea, which would slowly yield to it. If the muscles producing it remained at their new length the pressure would fall but if they continued their contraction the mesogloea of the body-wall would continue its extension. Muscular contraction will stop when the fibres reach a fifth of their maximum length (Batham and Pantin, 1951), which implies about a doubling of the circumference (Chapman, 1950).

The external forces to which the animal is subjected in life are difficult to assess but are probably not very great. When immersed in water and subjected to water currents, its drag is clearly small since a detached animal sinks rapidly in sea water when the force of gravity acting on it is slight, since its specific gravity is very near to that of its environment. The forces exerted by breaking waves on animal structures are hard to determine or to estimate, but, again, may be smaller than is commonly believed. For example, the egg cocoons of

Scoloplos armiger, pear-shaped gelatinous masses about 1 ml. in volume, are attached to the sand by a stalk which requires between 5 and 10 g. wt. to break it. These cocoons endure for some weeks and are swept four times a day by breaking waves which may be as much as 2 ft. high, yet the cocoons manage to keep their station and are not washed away (Chapman, Forrest, and Newell, unpublished observations). Sea anemones are clearly much tougher structures than are gelatinous egg masses, so their resistance to wave action is not surprising. For the most part, however, they seem to prefer not to be exposed to the full force of the waves. When deformed by an external force acting, as waves act, for only a short time, its action will fall within the time of elastic response of the mesogloea rather than that of permanent deformation. Thus the creature is well adapted by the physical properties of its connective tissues to withstand the forces, both of its own muscular actions and of the environment, to which it is exposed in life.

Another function of the mesogloea which has been pointed out by Batham and Pantin (1951) is simply the provision of a basis on which the muscle fibres are, as it were, mounted and which allows them very greatly to change their length. These authors have shown that the outer layers of the mesogloea on which the muscle fibres lie are much less extensible or deformable than is the central part and that the buckling of the muscular layer takes place to accommodate the fibres when contraction and diminution of volume take place. The mesogloea can therefore be regarded as *itself* having a fixed volume even in those anemones which show great changes of the coelenteric volume.

Finally, there is the possibility that the mesogloea may play the part of a food reserve. In the ctenophore *Pleurobrachia pileus* which, of course, is not a cnidarian coelenterate, the size of the jelly rapidly diminishes on starvation and the animal becomes shrunken and distorted within a week. Huxley and de Beer (1924) reported similar changes, accompanied by dedifferentiation, in starved *Aurelia*. It would be interesting to know if this is a general feature of the phylum Coelenterata.

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The Ability to Differentiate, and the Size of Regenerated Cells, after repeated Regeneration in *Spongilla lacustris*

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SUMMARY

The findings presented here have a bearing on regeneration in general. They show that even in simply organized animals, the number of totipotent cells which are able to differentiate decreases as the individual ages. In older specimens it takes longer for the totipotent cells to differentiate than in younger ones; at the same time the nuclear and cytoplasmic volume of these cells is reduced.

The expanded basal epithelium of sponges germinating from gemmules is an organ necessary to establish the tension in the body which is indispensable for the functioning of the sponge. Sponges that have germinated from gemmules can be forced to regenerate a basal epithelium. The materials for this regeneration is furnished by the archaeocytes, which are embryonic, totipotent amoebocytes. The number of archaeocytes that are able to perform this regeneration decreases with time, i.e. as the differentiation of the entire body proceeds.

On the other hand, the ability of the sponge to expand repeatedly on its own basal epithelium after being pushed away from it is limited only by the onset of cytolysis.

The ability of the archaeocytes to regenerate new *typical* basal epithelial cells is reduced after repeated regeneration.

The size of both nucleus and cytoplasm is reduced more and more during repeated regeneration. The nucleo-cytoplasmic ratio is thus kept fairly constant.

INTRODUCTION

An organism having the ability to regenerate lost parts must draw upon a stock of undifferentiated cells to restore its integrity when injured. This stock is a limiting factor in the organism's ability to regenerate. It is still an unsettled question whether such undifferentiated cells have always been embryological in character or whether some of them, owing to forces released by mutilation, attain this primitive state after dedifferentiation from more specialized conditions. Evidence is, however, more and more in favour of the first alternative. In lower organisms, such as sponges, the regenerative power seems to be almost unlimited; it has been assumed that the archaeocytes (amoebocytes wandering in the exoplasm of the sponge (Brøndsted, 1936)) are able to transform themselves almost unrestrictedly into any kind of sponge-cell. In the earlier paper (1936) it was shown (and this was independently confirmed by Brien (1937)) that after dissociation of the sponge body by pressing it through boulting cloth, the four main cell-types again participate in building up small functioning sponges, taking up their proper positions in relation to one another. At that time I pointed out that we cannot expect the

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necessary proportion between the four cell-categories to be established in the small reunited bodies that have come into being by random assembly o wandering cells; it must therefore be assumed that the apparently least-differentiated category of cells, the archaeocytes, are able to differentiate into missing cells of other categories.

If this viewpoint is correct, the question arises: Is the presumed potency o the archaeocytes unlimited? The present paper is an attempt to answer this question.

In the autumn the freshwater sponges, *Spongilla*, *Ephydatia*, &c., break up into gemmules, 0·3–0·8 mm. in diameter; these consist only of yolk-laden archaeocytes surrounded by a horny capsule. In the spring the archaeocytes creep out through a preformed micropyle in the capsule, and slide out on to the skeleton, which consists of siliceous spicules cemented together with a horny substance, the spongin. The archaeocytes differentiate in a few days. The process has been described by several authors, especially by Brien (1932), who gives full evidence that the archaeocytes differentiate into other cell-types. I have shown (Brøndsted, 1943) that a certain tension in the little organism is necessary to ensure organization of a functional water-canal system. This tension is set up by the basal epithelium, which spreads out on the substratum with exceedingly flattened cells that glue themselves on to it. By means of the electron microscope, Brøndsted and Carlsen (1951) have shown that this very strong adhesive power of the cells of the basal epithelium is due to a submicroscopical cortical feltwork of fibrils (presumably of spongin), built by the cells that cover the substrate.

It is possible to push away a little sponge from its substratum in such a manner that the basal epithelium remains upon it (Brøndsted, 1943); the sponge will round up in a few minutes and spread out again in the course of 1 or 2 days when placed on another slide. If, however, the rounded-up sponge is replaced on its own basal epithelium it will again spread out upon it, and with a speed many times greater than that of a sponge placed on a clean slide (Brøndsted, 1943).

We see here the counterpart of the well-known fact that the epithelial cells bordering a wound rapidly spread out and try to cover the wound. In our experiments, the remaining basal epithelium represents the wound and the body-cells have to spread out to cover it, and this they do with great speed. When the wound is closed, the integrity and tension of the sponge is restored, and very soon the proper organization for functional activity is attained.

The situation is different when the sponge, after being pushed away from its basal epithelium and having rounded itself up, is placed on the clean surface of a slide. Here the situation resembles that of the germinating gemmule, for a new basal epithelium has to be made.

The question arises: Are archaeocytes able to differentiate into basal epithelial cells indefinitely (or as long as the cells are able to move about)?

EXPERIMENTS

Preliminary investigations showed that 4 expanded individuals were able to make new basal epithelia 5 times each when pushed away soon after expansion terminated, whereas only 4 individuals out of 12 were able to make new basal epithelia 3 times when they had been expanded for some days and had begun their natural functions (the oscula and water-canal system had developed). The other 8 individuals, however, were still very much alive; they were in the state of globular cell-aggregates, wandering round on the slides without ability to expand.

A more extensive experiment was planned to study the now obvious question whether a time factor governs the ability of the sponge to regenerate the kind of differentiated cells which make up the basal epithelium.

Fifteen gemmules were brought to germinate on 3 slides, 5 on each. The first 5, group A, were pushed away from their basal epithelium and placed on new slides as soon as they had expanded fully. The next group, B, was allowed to differentiate for 2 days after expanding before they were pushed away. The third group, C, was pushed away after differentiating for 4 days; the sponges had at that time differentiated fully and were functioning. Four slides with several gemmules on each served as controls. The sponges were pushed away on the fourth day after expanding, when they were functioning fully; they were placed back on their own basal epithelia. When a sponge had expanded after being pushed away, it was again pushed away to test its expanding force. Table 1 gives a survey of the results.

The experiments show that the ability to make new basal epithelia diminishes rapidly when the sponges have had time to differentiate before they were

TABLE 1*a*

Survey of the ability of gemmules to make new basal epithelia at different times after the first expansion (hence at progressive stages of differentiation). After the sixteenth day no more expansions occurred

	Days from beginning of the experiment																				
	0	*1*	*2*	*3*	*4*	*5*	*6*	*7*	*8*	*9*	*10*	*11*	*12*	*13*	*14*	*15*	*16*	*17*	*18*	*19*	*20*
A1	exp. o	+	+	+		+	+		+						+						
A2	exp. o	+		+			+				+						+				
A3	exp. o	+		+	+				+				+								
A4	exp. o	+						+	+			+									
A5	exp. o	+	+	+	+				+		+		+								
B1	exp.		o		+					+											
B2	exp.		o					+			+										
B3	exp.		o		+				+	+			+								
B4	exp.		o		+			+	+				+		+						
B5	exp.		o														+				
C1	exp.				o																
C2	exp.				o																
C3	exp.				o		+							+							
C4	exp.				o																
C5	exp.				o																

exp. = first expansion. o = first removal from basal epithelium. + = expansion after every removal; the new removal took place when expansion was completed.

TABLE 1*b*

Controls to 1a. Expansion after removal and replacement on basal epitheli The individual gemmules have not been followed up. The figures are numbers c expanded specimens after every new removal. After the sixteenth day no mor expansions occurred

Control slides with	Days from beginning of the experiment																				
	0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	2
10 spec.	all exp.				0	9	8	8	7	7	7	6	5	5		4	3				
9 spec.	all exp.				0	9	9	9	8	9	9	9	9	9		9	8				
14 spec.	all exp.				0	14	14	14	14	14	13	14	13	12		7	7				
10 spec.	all exp.				0	10	10	10	10	10	10	10	9	10		8	6				

pushed away from their basal epithelium; as Brien (1932) shows, the variou differentiated cell-types (choanocytes, canal cells, spicule-forming cells) ar derived from the archaeocytes in the gemmule, and therefore in the function ing sponge the original stock of archaeocytes has been drawn upon heavily The 5 specimens of group A were able to rebuild a basal epithelium 28 times which gives an average of nearly 6 per individual. The specimens of group show an average of about 3 per individual, and those of group C only an averag of less than 0·5. The controls were able to expand on an average 7 times pe individual; but this figure is in reality too low because several of them were in a state of dissolution. The experiments indicate that the ability to produc a certain category of differentiated cells diminishes with the age and state o differentiation of the sponge in regeneration.

The question presents itself whether the ability to regenerate is correlate with the size of gemmules, i.e. the number of cells in the organism.

Fifteen specimens of various sizes were studied as to their ability to re generate new basal epithelia. Fifteen gemmules regenerating on their own basal epithelia served as controls. All individuals were pushed away from thei basal epithelia after they had differentiated (4 days).

TABLE 2

The ability of gemmules of various sizes to make new basal epithelia 4 days afte the first expansion. The size of the gemmules is indicative of the number of cell constituting the gemmule. The experiment shows that the number of cells is no correlated with ability to differentiate new basal epithelia

Size of gemmules in μ .	300	380	400	450	500	500	550	600	600	600	600	600	650	650	700
Number of expansions .	4	0	0	0	3	0	1	4	5	0	0	6	0	5	1

Controls. Here the gemmules had also expanded 4 days before they were removed but after removal they were placed on their own basal epithelium; therefore the did not need to differentiate new epithelia. Size of gemmule (and therefore th number of cells) is not correlated with ability to expand

Size of gemmules in μ .	350	350	400	400	450	450	450	500	500	550	550	600	600	650	700
Number of expansions .	10	11	11	11	10	10	11	9	11	10	11	9	9	10	10

It appears from this experiment that the size of the individual, and therefore the number of cells, is not correlated with the ability to differentiate basal epithelial cells. Furthermore, most controls that are made to expand on their own basal epithelium can do this up to 11 times each, and perhaps even more; if they are not cytolysing they can do this regardless of their size.

The typical basal epithelial cell has the appearance shown in fig. 1. Apart from the extremely flattened shape and the vacuolization in the cytoplasm around the nucleus, the basal epithelial cell can be recognized by its lack of nucleolus.

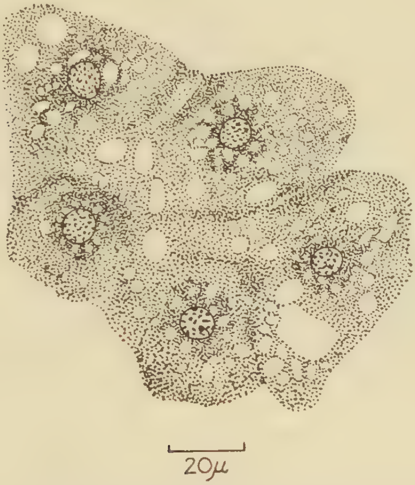

FIG. 1. Five cells of the normal basal epithelium of *Spongilla lacustris*.

The basal epithelia of the sponges in the above-mentioned experiment were fixed in Zenker's fluid and stained with haemalum. A count of the better part of the first basal epithelium of sponge no. 13 in table 2 showed 14 cells provided with nucleoli out of 309 cells, i.e. 4·5 per cent. In the sixth and the last basal epithelium from the same sponge, 73 cells out of 186 cells, i.e. 39 per cent., had a nucleolus. Fig. 2 shows basal epithelial cells after five regenerations in the same individual as fig. 1.

In another individual, no. 15, from the same experiment, the first basal epithelium had 7 cells each with a nucleolus out of 557 cells, i.e. 1·3 per cent., whereas the third basal epithelium had 45 cells with nucleolus out of 137 cells, i.e. 33·5 per cent. In still another experiment, one individual in the first basal epithelium had 4·8 per cent. cells with nucleolus, but 13·2 per cent. in its third epithelium; and in a fourth individual the corresponding figures were 3·3 per cent. and 10·3 per cent.; in a fifth the figures were 1·2 per cent. and 10 per cent.

A question of considerable interest is whether or not the size of the regenerated cells diminishes after repeated regeneration. The sponge considered in this paper lends itself to the solution of this problem.

An extensive account of the size of the nucleus in the basal epithelium i the living state and after fixation by some 30 different methods will be give elsewhere (Brøndsted, A., and Brøndsted, H. V., in press). It will be show that the measurement of 100 nuclei is quite sufficient to give a reliable averag of their size; the influence of the fixatives on nuclear volume is also discussed In specimen no. 15 the nuclei in the first epithelium have a diameter averagin 8·78μ, in the third 8·41μ, and in the sixth 7·91μ; i.e. during six regenerativ processes, the diameter of the nuclei had diminished by about 10 per cent. and their volumes by about 30 per cent. In another specimen, no. 13, there is a decrease of nearly 50 per cent. in volume through six passages.

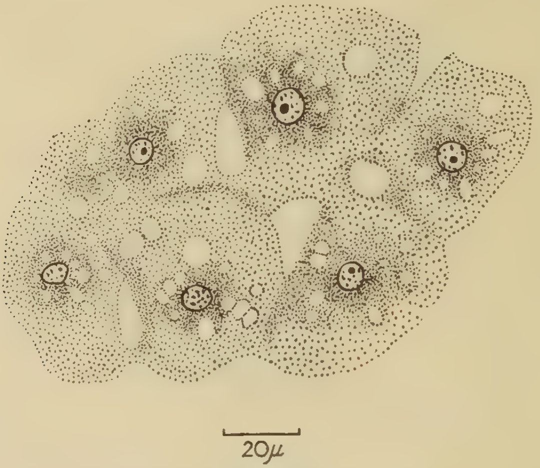

FIG. 2. Six cells of the basal epithelium of *Spongilla lacustris* after five regenerations. Th specimen was fixed and stained in the same way and for the same times as the specimen o fig. 1. Note the lighter appearance.

It is not only the nuclei that diminish in the course of repeated regeneration. The areas of the expanded basal epithelial cells were measured by counting the number of cells covering a certain area. In the first basal epithelium 939 nuclei were found; the area of each basal cell here averages 1959μ^2; whereas in the third, in which 1,174 nuclei were counted, each cell has an area of 1469μ^2, so that the cells have diminished their volumes by at least 25 per cent. during the whole regeneration period.

DISCUSSION

Several points in these investigations have a bearing on important problems of regeneration.

Even such simply organized animals as the sponges have rather a limited power of regeneration; the ability to regenerate differentiated cell-types is inversely proportional to the state of differentiation of the entire animal. That means that the organism attains its functional organization at the cost of embryonic developmental possibilities. This agrees well with the diminishing regenerative power of (for example) amphibian embryonic cells, which shift to new differentiation patterns; it is a well-known fact that the regenerative power diminishes from the early gastrula stage onwards: 'the doors are being closed', to use an expression of J. Needham.

The archaeocytes represent the stock of embryonic undifferentiated cells; as Brien has shown, these cells differentiate to the various categories of cells necessary to build a functioning sponge; a certain equilibrium between the number of cells of the various types is attained in normal development, but a certain stock of archaeocytes is available for new differentiation in spite of the fact that in sponges that are no longer able to expand, several archaeocytes are still found. There exists clearly a minimum amount of archaeocytes below which renewed differentiation to other cell types is not possible. It remains obscure what are the forces governing this equilibrium.

In our experiments the expansion of the sponge on its own basal epithelium resembles the phenomenon of the closing of a rather big wound; this process requires energy; this is apparent from the following fact. When rounded-up sponges at last fail to expand after they have been placed back on their own epithelium, they die, showing cytolysis; whereas sponges, which at last fail to expand on new slides when pushed away from their basal epithelium, are still alive for some days and able to move about; therefore they do not require as much energy as for expanding.

The ability of the archaeocytes to differentiate to true basal epithelium cells decreases with time. The first time basal epithelium is normally made, the archaeocytes very soon transform themselves, and in doing so lose their nucleoli; in repeated regeneration the transformation is harder to accomplish; this is seen from the fact that more and more cells still retaining their nucleoli are found when the basal epithelium is regenerated on later occasions. It is obvious that our material offers an opportunity of studying the question of cytochemical processes involved in, at least, one course of cell-differentiation. Investigations are proceeding in our laboratory.

The question whether by repeated regeneration the cells retain or diminish their size is, of course, of interest. The answer is important in connexion with the question whether the organism strives to maintain the vigour of some working cells at the expense of other cells or if all cells suffer alike under the strain. In the experiments with *Spongilla* it has been shown that all cells involved in regeneration diminish their size; i.e. all cells share the common burden.

The reduction in the size of the regenerating cells affects the nucleus as well as the cytoplasm. The measurements do not give precise information about the nucleo-cytoplasmic ratio, but they suggest that it remains constant. It is assumed that cyto-chemical investigations, together with those mentioned above, may prove fruitful for the understanding of some of the physiological interrelations between nucleus and cytoplasm. In this connexion reference should be made to the very interesting paper of Schrader and Leuchtenberger (1950).

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Macronuclear Regeneration in *Epistylis articulata*

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With one plate (fig. 5)

SUMMARY

The reorganization of the macronucleus from the vegetative micronucleus is described in *Epistylis articulata* (Ciliophora, Peritricha). Individuals which have accidentally been deprived of a macronucleus during binary fission develop a new one from the micronucleus. In such amacronucleate animals, the micronucleus divides into two, one of them growing into a macronucleus, the other remaining as the micronucleus. The phenomenon throws new light on the relative importance of the two parts of the ciliate nuclear apparatus and brings into greater emphasis the undifferentiated nature of the micronucleus. It also establishes the possibility of *de novo* synthesis of DNA in the developing macronuclear rudiment.

INTRODUCTION

THE possession of two nuclei—the macronucleus and the micronucleus—is characteristic of all euciliates. Associated with this separation of the nuclear apparatus into two components, there is also a differentiation in their size, mode of division, and function. The necessity of either or both these nuclei for the normal life of the organism has been repeatedly stressed, but occasional instances where one or the other is absent have been reported, always with more or less conspicuous effects on the organism and its life cycle. For instance, Diller (1940) reported the occurrence of individuals of *Paramecium caudatum* without a macronucleus but regarded the condition as probably due to centrifugation, senility, or changes in nutritional and environmental conditions. In all cases, he thought, such animals died early. Sonneborn (1947) studied, from his stocks of *Paramecium aurelia*, individuals without one or the other nucleus; and while he discovered that those without a micronucleus could survive for considerable periods, animals without a macronucleus met with early death. On these findings he developed the view that while the macronucleus was essential to the vegetative life of the organism and could not be replaced when lost, the organism could do without a micronucleus for a considerably long period.

While making a study of *Epistylis articulata* it was found that amacronucleate individuals turned up in large numbers in certain colonies with such frequency as to merit a closer examination of their origin as well as their fate. Several hundred such individuals were found in our stocks and a minute study revealed certain interesting features.

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MATERIAL AND METHODS

Colonies of *Epistylis articulata* (From.) were obtained from sewage waters about five miles from Bangalore. Among the dominant organisms met with when raw sewage is aerated are peritrichous ciliates of the genera *Vorticella*, *Epistylis*, *Carchesium*, and *Zoothamnium*. This fact was made use of in obtaining large supplies of material which was brought to the laboratory for further examination and treatment. The material was available abundantly throughout the year.

The material was fixed in hot Schaudinn's, Bouin's, and Carnoy's fluids and was stained in haematoxylin, Feulgen, toluidine blue, and Unna's methyl green pyronin mixture. All the diagrams and photographs illustrating this paper are based on Feulgen preparations.

OBSERVATIONS

Amacronucleate individuals of *Epistylis articulata* occur among the normal members of the colony scattered at random and without any special arrangement or distribution. They occur at all levels of the colony. They can be easily recognized by their smaller size and by the absence of the conspicuous macronucleus, which, in this species, is cylindrical and bent, occupying a considerable amount of space in the cytosome.

The amacronucleate condition results from a slight upset of the process of binary fission. In *E. articulata*, binary fission normally starts with the mitotic division of the micronucleus. Long after the daughter micronuclei have separated and moved apart, the macronucleus stretches across the animal at right angles to the cleavage furrow, which, beginning at the peristome, passes towards the stalk. Eventually the macronucleus is cut into two approximately equal parts which are shared by the two daughter organisms.

In the production of the amacronucleate animal, the micronucleus divides in the usual manner, but the macronucleus, instead of being cut into two parts, remains entirely within one of the daughter cells. The amacronucleate individual is much smaller, while the one with the macronucleus is apparently not different from the normal animals of the colony, except perhaps for the visibly larger macronucleus (fig. 1, A, B, and C).

The amacronucleate animal has an interesting future. For a considerable time it remains quiescent and inactive: it is doubtless unable to function as a normal animal. It seems incapable of going through even the one or two fissions that *Paramecium* appears able to achieve (Sonneborn, 1947). The duration of such inactive existence can be easily estimated, in a branching colony like that of *Epistylis*, by a study of the sister individual which has the macronucleus. This latter, it was observed, proceeds with its normal existence, feeding, and reproducing by binary fission. Three or four generations of individuals are produced by the sister animal with the macronucleus while the amacronucleate one has remained, during this period, a small and quiescent organism. It is doubtful if it feeds. It certainly does not seem to grow. It does not divide.

Soon, however, the micronucleus of the amacronucleate animal divides by mitosis (fig. 2). We have not been able to detect any difference between

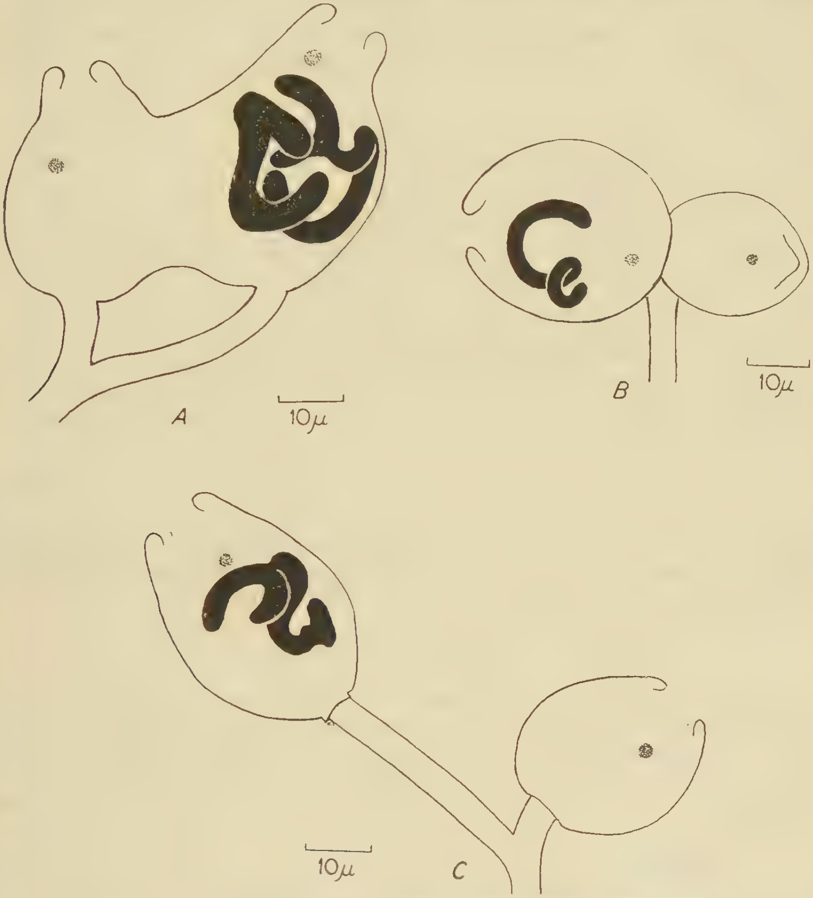

FIG. 1. Formation of amacronucleate animal. An upset in binary fission results in the formation of two individuals, one of which lacks the macronucleus. A, fission is in progress. The micronucleus has divided but the macronucleus is included in one of the animals. B, later stage. Fission complete. The amacronucleate animal is visibly smaller. C, later stage. The difference in the length of the stalk of the two animals is clear.

mitosis of the micronucleus in a normal and an amacronucleate animal; probably there is none. The two daughter nuclei are at first similar in size but soon one of them enlarges and becomes the macronucleus. Even in its initial stages of enlargement, the macronuclear rudiment or primordium shows a striking difference in structure and we have been able to trace the entire process of its differentiation. The micronuclear rudiment, like the micronucleus of the vegetative animal, remains a uniform lightly stained body.

One of the characteristics of the micronucleus is that it shows a smooth homogeneous appearance in preparations made in a variety of ways: haematoxylin, borax carmine, Feulgen, and methyl green. The macronuclear rudiment, on the other hand, shows in its earliest stages of differentiation, coarse deeply staining blocks of chromatin (fig. 3, A). As the rudiment enlarges further it becomes fainter.

The further history of the macronuclear anlage is of interest. It continues to increase in size, and at one time occupies a considerable space in the cell, pushing the micronucleus to a side (fig. 3, B and C). At this stage it remains a faintly staining body. Later, it gradually shrinks in size, and with this shrinkage is associated an unmistakable acquisition of basiphilia, until the macronucleus assumes the final condition characteristic of the vegetative macronucleus. At first spherical or oval, it soon becomes elongated and cylindrical and assumes its definitive form (figs. 4, A and B, and 5).

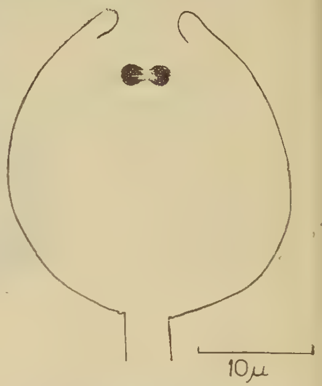

FIG. 2. Amacronucleate animal. Division of the micronucleus is in progress.

With the full development of the macronucleus and the completion of the nuclear equipment, the animal behaves like a normal organism and becomes indistinguishable from it.

DISCUSSION

The regeneration of the macronucleus from the micronucleus of a vegetative individual has never before been reported in any ciliate. That after conjugation or autogamy, the synkaryon possesses this property is well known. That this is shared by the vegetative micronucleus is clear from the above observations.

The nearest parallel we have to this phenomenon is in endomixis. This process was first described by Woodruff and Erdmann (1914) in *Paramecium aurelia*, but it has been noticed in a number of other ciliates too (see Woodruff, 1941, for a review of the subject). Recently some doubt has been expressed on the occurrence of endomixis, especially in *P. aurelia*. Sonneborn (1947) has never met with it in his stocks. The cytological evidence is also not conclusive. However, the resemblance between endomixis as described by Woodruff and the vegetative regeneration of the macronucleus described in this paper is striking. In both cases it is the micronucleus (or one of its division products) of a vegetative organism that produces the macronucleus. There is no fusion of nuclei involved, either in single animals as in autogamy, or in pairing animals as in conjugation.

But the parallel ends there. In endomixis there are two complications which are absent in the macronuclear regeneration described in this paper.

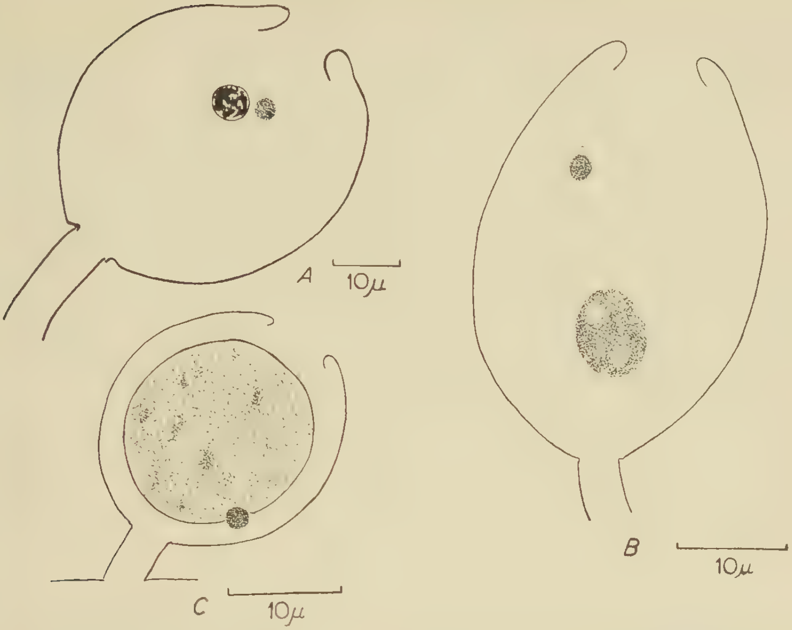

FIG. 3. Differentiation of the macronucleus. A, an early stage, showing the macronuclear rudiment with deeply staining chromatin. B, later stage in differentiation. The enlarging pale macronuclear rudiment is clear. C, stage showing maximum enlargement of the macronuclear rudiment. The micronucleus lies to one side.

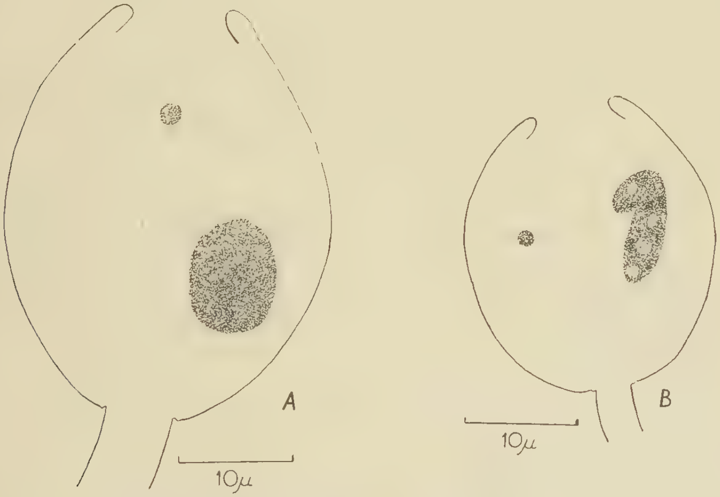

FIG. 4. A, differentiation of the macronucleus. The rudiment has visibly shrunk in size and stains deeply. B, an early stage in the acquisition of the definitive form of the macronucleus.

First, the micronucleus in endomixis passes through divisions which recall the progamic divisions in conjugation and autogamy. Secondly, the animal has, in endomixis, a macronucleus, which, however, breaks up and is absorbed in the cytoplasm in much the same way as in conjugation and autogamy.

Doubts have been expressed, on both cytological and genetical grounds, of the occurrence of endomixis in ciliates. These refer particularly to the two divisions of the micronucleus immediately before differentiation. Their occurrence in conjugation and autogamy is of significance in that they are meiotic, reducing the diploidy of the micronucleus of the vegetative animal to haploidy, which is restored to diploidy by syngamy. In the absence of syngamy, the divisions could not be meiotic and it is difficult to imagine the necessity for these divisions in endomixis. It seems likely, as Diller (1936) remarks, that Woodruff and Erdmann were mixing up stages of autogamy and hemixis and have lumped them together under endomixis. On this analysis, the behaviour of the micronucleus described in this paper seems simple and straightforward. The single division of the micronucleus must be a mere mitotic division and both the rudiments are diploid. The growth of one into a macronucleus is a subsequent phenomenon not affecting the essential nature of the ploidy of the nucleus.

The other matter of the breaking up of the macronucleus characteristic of conjugation, autogamy, and endomixis raises a problem of even greater interest—the origin of the desoxyribose nucleic acid (DNA) of the developing macronuclear anlage. It is clear from a number of earlier studies (Seshachar, 1947, 1950; Seshachar and Dass, 1951; Seshachar and Srinath, 1946 and 1947) that the ciliate macronucleus has a large amount of DNA in it. The synthesis of DNA in the developing macronucleus after conjugation has formed a subject of considerable interest, and more than once it has been suggested that the DNA of the new macronucleus must in some manner come from the old and degenerating macronucleus and its break-down products (Seshachar, 1947; Baker, 1948; Glass, 1949). There has been some indirect evidence for this transfer in the form of a gradual degeneration and disappearance of the fragments of the old macronucleus as the new macronucleus is acquiring its DNA. In *Vorticella convallaria* (Seshachar and Dass, 1951) there is a gradual reduction in the number of fragments, associated with the acquisition of DNA by the rudiments. In *Epistylis articulata* (Seshachar and Dass, in press) the fragments of the old macronucleus, which in the early stages are uniform, brilliantly

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Fig. 5 (plate). Photomicrographs showing the formation of amacronucleate animal and the differentiation of the macronucleus in it.

A, formation of amacronucleate animal.

B, typical amacronucleate animal.

C, early stage in the differentiation of the macronucleus. The difference between the two rudiments is clear.

D, macronuclear rudiment in the process of enlargement.

E, macronuclear primordium at maximum enlargement. The micronucleus lies to one side.

F, macronuclear primordium has shrunk in size and stains deeply. Compare with D.

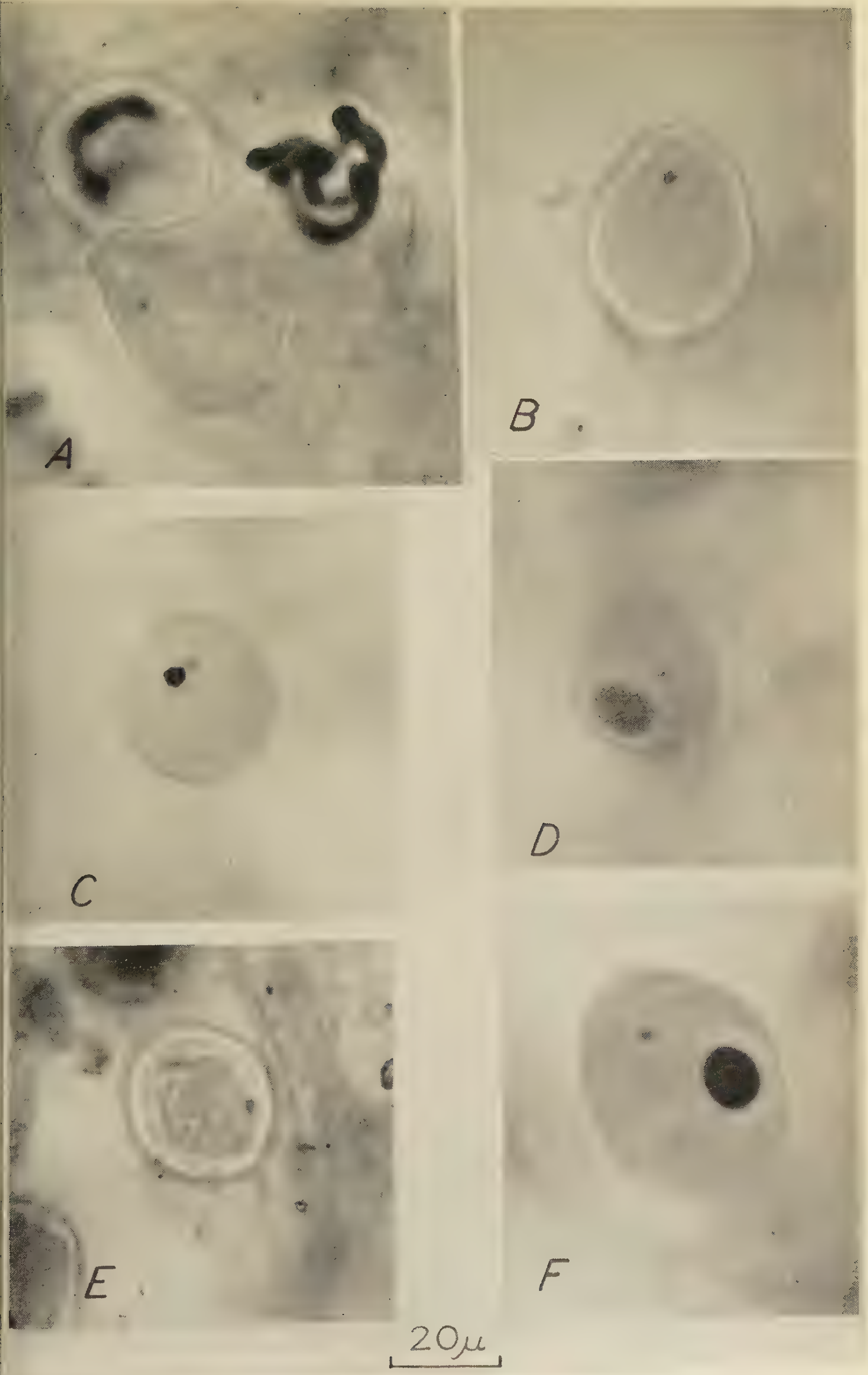

Fig. 5
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Feulgen-positive bodies, gradually develop Feulgen-negative spaces within them, until each fragment finally becomes a Feulgen-negative body. On the basis of a specific staining technique like that of Unna-Pappenheim (pyronin methyl green) we have reasons to believe that this involves a fundamental change in the nucleic acids of the fragments the DNA being gradually replaced by RNA (ribose nucleic acid). Then again, there is abundant evidence of a more general nature, in a number of ciliates, where, concomitantly with the development of the new macronucleus, there is a gradual dwindling and disappearance of the old. Around these observations has developed the idea that the synthesis of DNA in the new macronucleus must in some manner be connected with the disintegration and disappearance of the old, and a causal connexion must exist between the two, however undemonstrable cytologically it might be. It seems, however, not necessary to believe that a *de novo* synthesis of DNA in the new macronucleus is impossible. Actually, considerable *de novo* synthesis of DNA has been demonstrated by photometric methods in the developing macronucleus (after conjugation) of *Chilodonella* (Seshachar, 1950).

The observations recorded in this paper seem to put the entire problem of the *de novo* synthesis of DNA in the macronucleus beyond reasonable doubt. There is no macronucleus or its fragments to cloud the issue.

We also see another interesting resemblance between the present instance of macronuclear regeneration in vegetative individuals of *Epistylis articulata* and the development of the macronuclear rudiment in *Chilodonella uncinatus* after conjugation, described by one of the authors (Seshachar, 1950). In both cases, the initial stage of macronuclear differentiation consists in an enlargement of the rudiment to considerable dimensions, without acquisition of DNA at a corresponding rate; so it appears as a large faintly staining body, occupying much of the cell space (figs. 3, C and 5, E). Later it shrinks in size, meanwhile acquiring DNA and the final form of the macronucleus. The initial process involving a conspicuous enlargement of the rudiment to great dimensions, occurring as it does in two very different circumstances (one after conjugation and the other in vegetative reorganization), is one of the most striking phenomena in the nuclear reorganization of ciliates, whose precise significance is not clear, but whose interest is not, on that account, any the less.

Then there is the final question of the relative importance of the two parts of the ciliate nuclear apparatus to the life and life-history of the animal. Sonneborn (1947) believes that in *Paramecium*, an individual without a macronucleus is unable to survive long, while it is capable, deprived of the micronucleus, to live for a considerable length of time. In the light of the observations set forth in this paper, it seems necessary to modify this concept, at least in so far as it relates to peritrichous ciliates. The lost or missing macronucleus *can* be regenerated by the vegetative micronucleus, outside the conventional processes of reproduction and reorganization—conjugation, autogamy and endomixis. This, however, does not take away any of the importance attached to the role of the macronucleus in vegetative life; rather it adds to it.

But it also emphasizes the role of the definitive micronucleus, not the synkaryon alone, in the development of the macronucleus. The macronucleus has been accepted for a long time as a highly organized body irreversible in its differentiation, while the micronucleus is comparatively undifferentiated and capable of producing both nuclei. We had been led to believe that only the synkaryon was in this protean condition; it now seems necessary to regard the formed micronucleus also as sharing this character.

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The Ligament of *Pecten*

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SUMMARY

The ligament of *Pecten maximus* consists of two layers, the outermost situated along the extensive dorsal margin of the valves, the inner between them at the umbo. The former, composed largely of tanned proteins, is laminated and is somewhat similar to the outer layer of other ligaments. The inner layer is divided into three parts, a large central non-calcified structure and two lateral calcified regions attaching the former to the valves. The lateral parts resemble the entire inner layer of the ligament of most other lamellibranchs, which is generally uniformly calcified. The central region, consisting mainly of a tanned protein complex, is gelatinous in appearance and is characteristic of *P. maximus* and associated species. This type of structure is particularly suitable for the rapid and frequent opening of the valves, and its relatively high efficiency may be largely attributed to the non-calcified region of the inner layer.

INTRODUCTION

THE shell of lamellibranchs consists of a pair of valves joined together dorsally by a horny structure termed the ligament. This structure causes the valves to open when the adductor muscles relax.

The morphology, development, and nature of the ligament of various lamellibranchs has recently been described (Trueman, 1949, 1950*a* and *b*, 1951, and 1952), and the fundamental structure of the ligament and mantle discussed (Owen, Trueman, and Yonge, 1953). Apart from a brief description of some aspects of the ligament of *Pecten* (Newell, 1937 and 1942), little attention has been given to the ligament of this genus, which is remarkable in view of the interest in its adductor muscles (e.g. Bayliss, Boyland, and Ritchie, 1930) and in the evolution of the swimming habit in the Lamellibranchia (Yonge, 1936).

The mechanical properties both of the ligament of *P. maximus* and of that of the rather similar *Chlamys opercularis* have recently been examined (Trueman, 1953). The present paper gives an account of the structure and nature of the ligament of these lamellibranchs and discusses their form in relation to their mechnical properties.

GENERAL STRUCTURE

The ligament of *P. maximus* is situated between the dorsal regions of the valves (fig. 1) and extends both in front of and behind the umbo. It may therefore be referred to as amphidetic.

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The ligament of many lamellibranchs consists of the following four layers the periostracum, covering the outer surface of the valve and ligament, th fusion layer, and internal to these the outer and inner layers of the ligamen (Owen, Trueman, and Yonge, 1953). In *P. maximus* the periostracum is very thin and apart from its attachment to the ligament laterally need not be considered.

The inner layer of the ligament of *Pecten* is a large internal structure comparable with that of other lamellibranchs. It is of a dark brown colour, is situated between the valves in the subumbonal region (fig. 1), and is joined to the valves in the ligament pits or resilifers. It is pyramidal in shape with a base that bulges ventrally between the valves, particularly when they are closed.

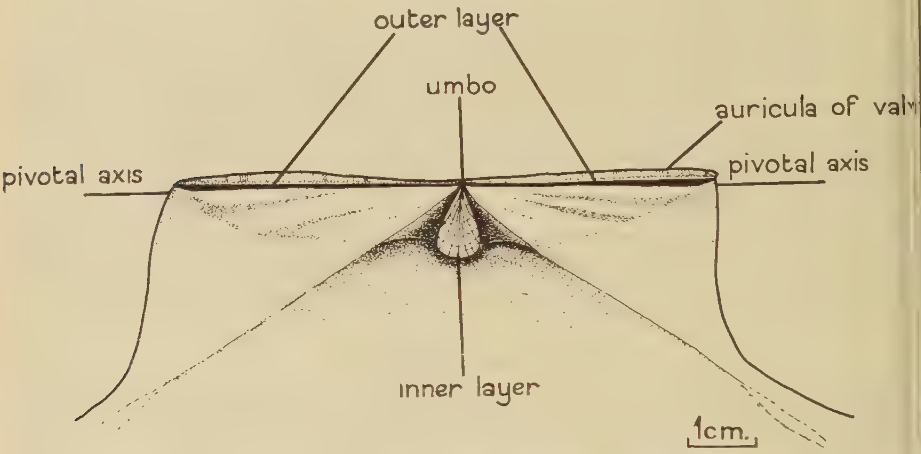

FIG. 1. Interior of the dorsal region of the right valve of *P. maximus* with the ligament cut in longitudinal section. The position of the pivotal axis is indicated (approximately natural size).

In addition to the inner layer and the periostracum, a single elongate layer (fig. 1) of a brown colour unites the dorsal margins of the valves which are extended antero-posteriorly in *Pecten* to form the auriculae. This single layer might represent either the fusion layer or the true outer layer, for convenience of reference it may be called the outer layer, but the interpretation of this layer will be discussed below.

When the elongate outer layer is viewed in transverse section, it appears approximately rectangular in shape, the width between the valves being much smaller than its thickness (fig. 2). Sections of small specimens of *Chlamys opercularis* (6 mm. overall length) were used for the examination of this layer. In transverse sections of the latter near the anterior and posterior margins of the outer layer, the periostracum may be seen attached to the dorsal edges of the ligament. There is no differentiation of the outer layer when ordinary staining techniques are used. In these specimens of *Chlamys* the more recently formed outer layer at the anterior and posterior margins stains pink in Mallory's triple stain. Sections of the outer layer of the adult *P. maximus* appear as dark structures, red when stained by Mallory's triple stain; sections of the more central region of the outer layer of the ligament of *Chlamys* are somewhat intermediate between the two conditions.

In such transverse sections as those of *Chlamys* the outer layer is only faintly birefringent. This is in contrast to the marked anisotropic properties of this layer in certain other lamellibranchs, e.g. *Tellina tenuis* (Trueman, 1949), where the optical properties appear related to the tensile stress to which the outer layer is subjected. The outer layer of the ligament is laminated, the laminae appearing in transverse section to be parallel to its ventral surface (fig. 2). Each group of lamellae extends to the outer surface at the anterior or posterior margin of the layer, where they are thicker than those more centrally (fig. 4, B). Growth of the outer layer of the ligament of *Pecten* takes place both by elongation and by the simultaneous thickening of the more central outer layer, which occurs in *Pecten* in the absence of an extensive inner layer. As the shell grows the more dorsal part of the outer layer frequently becomes worn away and is replaced by more ventral secretion.

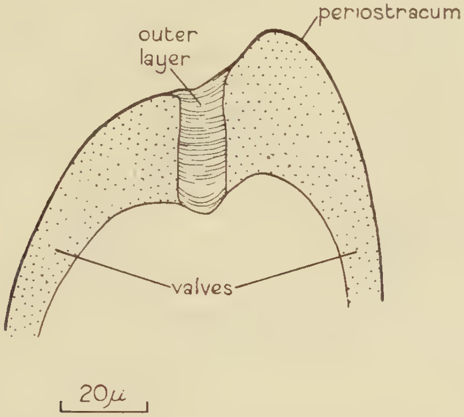

FIG. 2. Transverse section of the outer layer of the ligament of *C. opercularis* showing the characteristically laminated appearance. In this figure and in the one following the probable position of the valves is indicated by the stippled area.

Transverse sections of the outer layer of the ligament of small specimens of *C. opercularis* (1·5 mm. overall length) were examined with particular care regarding its differentiation into fusion and outer layers. It is considered that if there was such differentiation it should be apparent in sections of an early post-larval stage. The fusion and outer layers of the ligament of many lamellibranchs, e.g. *Tellina* or *Mytilus*, may be easily differentiated in sections stained by Mallory's triple stain, but when sections of the small specimens of *C. opercularis* were stained in this manner only the laminae could be seen. Thus the region termed the outer layer appears to consist of a single kind of layer.

The inner layer of the ligament may be divided into three parts: a large non-calcareous central region, dark brown in colour and of rubber-like consistency, and two smaller lateral calcified regions attaching the former to the valves. The shape and relationship of these parts to each other and the oute layer are best understood by reference to fig. 3. The inner layer of the ligamen of many lamellibranchs, e.g. *Mytilus*, *Ostrea*, or *Tellina*, is calcified. Th central part of that of *Pecten* or *Chlamys* is not calcified although it show structures similar to the inner layer of the other genera. The two regions o the inner layer may be easily differentiated by use of Mallory's triple stain which colours the central part red and the lateral parts blue.

In transverse and longitudinal sections the inner layer presents a laminate appearance, the lamellae being nearly parallel to the ventral growth surface o

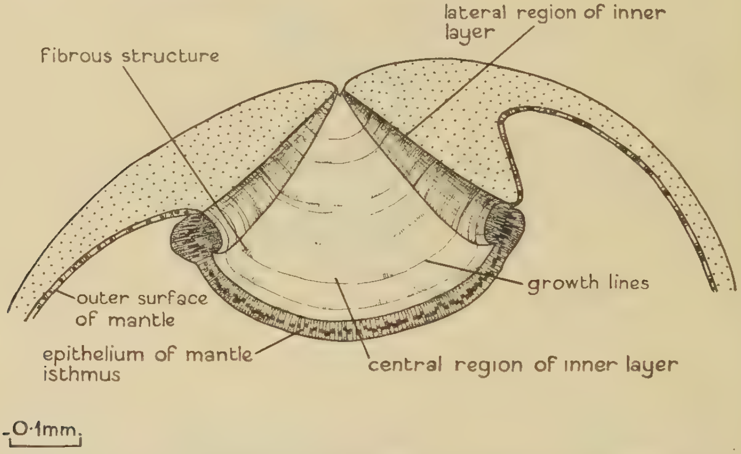
lateral region of inner layer
fibrous structure
growth lines
outer surface of mantle
epithelium of mantle isthmus
central region of inner layer
0·1mm.

FIG. 3. Transverse section through the centre of the inner layer of *C. opercularis* showing th structure of the central and lateral regions as described in the text. The outer layer has become worn away and the inner layer is slightly fractured dorsally.

this layer (fig. 3). These lamellae probably represent growth stages and correspond to the structures previously described as the growth lines of the inne layer of the ligament of other lamellibranchs, e.g. in *Tellina tenuis* (Trueman 1949, figs. 3 and 4). Examination of hand sections of the central region of the ligament of *P. maximus* clearly demonstrates the presence of fibres whose main axis lies normal to the ventral growth surface of the ligament and to the growth lines (fig. 3). The growth lines may be traced into the lateral calcified regions, where they are particularly prominent, indicating corresponding growth stages in all parts of the inner layer. In transverse hand sections of the lateral regions a well-marked fibrous structure may be observed, which appears similar to that of the central region. The structure of the lateral regions is very much more noticeable than that of the central, the latter being relatively amorphous.

The lateral parts of the inner layer of *Pecten* are similar to the complete inner layer of other lamellibranchs. This suggests that they represent the more typical part of the layer, the central region being a modification characteristic of this type. The inner layers of *Chlamys*, *Amussium*, and *Hinnites*

Yonge, 1951) are similar to that of *Pecten*, so that the modified inner region of the ligament appears characteristic of the Pectinidae.

The pivotal axis of the ligament lies immediately below the outer layer (fig. 1), but as the shell grows the axis tends to move ventrally. A small part of the inner layer thus becomes situated above the pivotal axis and subjected to tensile stress when the valves are closed, so causing slight splitting of the most dorsal region of this layer (fig. 3). The inner layer of the ligament of *Ostrea* and *Mytilus* (Trueman, 1951 and 1950*b*) has been shown to split similarly when subjected to tensile stress. As the fracturing of the inner layer occurs in the ligament of *Pecten*, so the dorsal part of the outer layer also splits. The outer layer may thus become completely worn away from above part of the inner layer (fig. 3). This phenomenon may be observed in large specimens of *P. maximus* and in these the replacement of the older parts of the outer layer by a more ventral secretion may easily be seen in longitudinal section. Above the outer layer of such specimens a region of previous attachment of the outer layer may be observed on the valve, showing parallel striations probably equivalent to the laminae of the outer layer now worn away. This occurs to a very much greater extent in *Hinnites multirugosus* (Yonge, 1951), apparently in response toc onstraint between rock surfaces when the upper valve is raised.

The ventral movement of the pivotal axis also occurs in *Ostrea* and appears to be characteristic of those lamellibranchs with internally situated ligaments, Dall (1895) observed that the longer the line covered by a functional ligament the more rigidly will the opening and closing of the valves be controlled. There are two methods of obtaining a long straight pivotal axis near the border of a rounded body: either the axis must pass inwards, or the outline of the body must be modified. The auriculae and the relatively long outer layer of the ligament of *Pecten* probably reduce the amount of ventral migration of the axis as compared with other lamellibranchs with internal ligaments.

THE MANTLE EPITHELIUM

The relation of the mantle to the various layers of the ligament, discussed recently by Owen, Trueman, and Yonge (1953) may be briefly noted. The periostracum covers the valves and ligament, although it is frequently worn away at the umbones. It is secreted from the periostracal groove, which is continuous around the mantle edge between the middle and outer lobes of the mantle. The fusion layer is secreted by the outer mantle lobe immediately below the periostracum in the dorsal midline. It is so called because it represents material secreted by the region of the outer mantle lobes which coalesce in very early post-larval life. This region is considerably elongated in many lamellibranchs and together with the periostracum forms a type of secondary ligament, which may extend both anteriorly and posteriorly to the outer and inner layers of the ligament.

The true outer layer of the ligament is generally secreted by the epithelium adjacent to the boundary of the mantle isthmus in the midline. The term ‘mantle isthmus’ is applied to the epithelium of the outer surface of the mantle, bounded peripherally by the outer mantle lobe, where this epitheliu forms a connecting neck of tissue between the two lateral lobes of the mantle The general outer surface of the mantle secretes the inner calcareous layer o the valves except at the mantle isthmus, where it secretes the inner layer of th ligament (fig. 4). The inner layers of the valves and ligament are thus closel related to each other.

The mantle of *Pecten* shows the characteristic periostracal groove and oute lobe of the mantle. Examination of the tissues of whole specimens shows

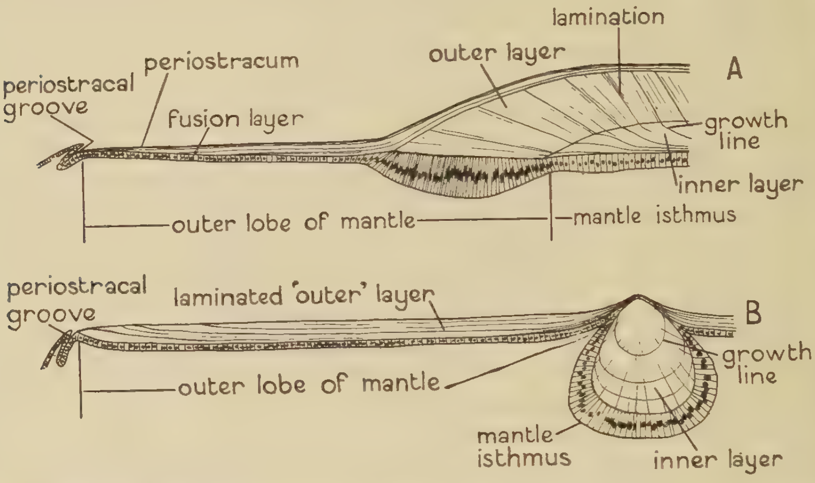

FIG. 4. Diagram of longitudinal sections through either anterior or posterior ends of lamellibranchs. A, basic type of ligament with periostracum, fusion, outer and inner layers. B, the ligament of *Pecten*. The epithelium of the mantle is shown below the ligaments. Not to scale

considerable depression in the dorsal region of the animal corresponding to the position of the inner layer of the ligament and representing the mantle isthmus (fig. 5). Between the outer lobe of the mantle and the central depression two thin ridges of epithelial tissue extend anteriorly and posteriorly along the dorsal midline (fig. 5). The epithelium of these ridges, although rather thicker than that adjacent secreting the valves, shows no localized regions of hyperdevelopment such as have been observed near the mantle isthmus in certain other lamellibranchs, e.g. *Tellina* (Trueman, 1949, fig. 8, *Ep*. 1*B*). The epithelium of the mantle isthmus is, however, considerably enlarged (fig. 3) as compared with the epithelium of the outer surface of the mantle secreting the inner layer of the valve.

THE NATURE OF THE LIGAMENT

Some observations on the nature of the ligament of *Anodonta* have been made by Trueman (1950*a*) and on that of *P. maximus* by Roycroft (1951). The outer layer of the ligament of *Pecten* is not particularly suitable material for investigation since only small portions are available, the central part of the inner layer being much easier to study. The term 'conchiolin' is frequently used to indicate the non-calcareous part of the molluscan shell. Consideration of whether or not there is any substance present in the organic matrix of the shell to which this term may specifically refer, is beyond the scope of this paper. 'Conchiolin' may be conveniently used to refer to the non-calcareous parts of the shell.

In appearance the central part of the inner layer is somewhat gelatinous, resembling a solidified 10 per cent. gelatin solution that has been tanned by being placed in a 1 per cent. benzoquinone solution for about 48 hours. The outer layer appears to be much more fibrous, and to be generally similar to that of the ligaments of other lamellibranchs. The median part of the inner layer of the ligament of *Pecten* is markedly different from its counterpart in other bivalves, largely because of the lack of calcification. The calcium carbonate content of the inner layer of the ligament of certain lamellibranchs has been approximately determined by the ignition of parts of the ligament. The residual ash was assumed to be calcium oxide and the calcium carbonate content of the inner layer estimated therefrom (Roycroft, 1951). The central part of the inner layer of the ligament of *P. maximus* contains only 0·6 per cent. by weight of calcium carbonate in comparison with that of the inner layers of *Lutraria lutraria* or *Anodonta cygnea* which amounts to 22 per cent. and 40 per cent. by weight respectively.

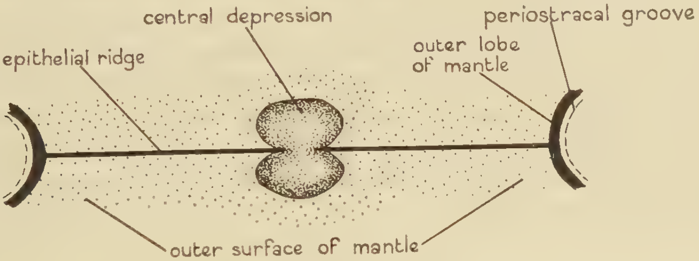

FIG. 5. Diagram of the dorsal region of the mantle of *Pecten* viewed dorsally.

The lateral parts of the inner layer appear both to be calcified and to have undergone some quinone-tanning, a condition rather similar to that of the inner layer found in many lamellibranchs, e.g. *Ostrea edulis*. The lateral parts might be described as being somewhat intermediate between the central region and the calcified shell.

Tests on the ligament of *Pecten* showed that it was extremely resistant to chemical action. It was soluble in concentrated alkali, however, and the chitosan test for chitin was negative. Positive results in tests for proteins, e.g. ninhydrin, biuret, indicated that they compose a large part of the ligament. The dark brown coloration of the outer layer and the central part of the inner layer suggest that the proteins had been affected by quinone-tanning.

Roycroft (1951) has investigated the type of protein linkage present in the ligament, by use of the methods described by Brown (1950). He placed sections of the ligament in a graded series of swelling agents, and found that the outer layer and the central part of the inner layer were soluble only in sodium hypochlorite solution and not in the other swelling agents. Solution only in sodium hypochlorite solution suggests the presence of aromatic tanned proteins (Brown, 1950).

The argentaffin reagent gave a positive reaction with both layers of the ligament; this may be considered due to the presence of polyphenols. Investigation of the precursors of the tanning agent in the tissues concerned with the secretion of the ligament, e.g. the ferric chloride test for orthodiphenols was carried out with inconclusive results. The presence of some lipoid material in the central region of the inner layer was also faintly indicated by the appearance of frozen sections stained by sudan black.

The main constituent of the ligament may thus be shown to be a tanned protein complex, fibrous in the outer layer and more gelatinous in the inner. The presence of the non-calcified region of the inner layer of the ligament of *Pecten* and *Chlamys* is unusual in comparison with other lamellibranchs, although a similar condition has been recorded in the ligament of *Nucula* (Trueman, 1952).

DISCUSSION

The structure and composition of the ligament of *P. maximus* and associated species has been outlined above. Whereas the ligamental structure of many lamellibranchs consists of the periostracum, fusion, outer and inner layers (fig. 4, A), in that of *Pecten* there is the periostracum and an inner layer but only one of the other two layers (fig. 4, B). From its location below the periostracum this layer could be termed the fusion layer but from its relationship to the inner layer it could be called the outer layer. Both the fusion and outer layers are secreted by the outer mantle lobe, the latter near the mantle isthmus by a band of rather enlarged epithelial cells in many lamellibranchs. No such area of epithelium has been observed in *Pecten*. The fusion layer is secreted from the more distal parts of the outer mantle lobe, which are fused together in very early post-larval life (Owen, Trueman, and Yonge, 1953). In many lamellibranchs, e.g. *Tellina* (Trueman, 1949), the fusion layer is relatively more extensive and thinner than the outer layer. The fusion layer is commonly laminated, the laminae generally being at their maximum thickness centrally, near the anterior and posterior margins of the outer layer (fig. 4, A).

Sections of the outer layer of the ligament of most lamellibranchs also show laminations, indicating stages in the secretion of the layer, corresponding to the growth lines of the inner layer (fig. 4, A).

In *Pecten* the outer layer is laminated, the laminae being at their maximum thickness peripherally (fig. 4, B). The fusion and outer layers, in contrast to the inner layer, are somewhat similar in structure and nature, and their main structural differences possibly result from their different rates of secretion.

If the so-called outer layer is actually the fusion layer, the true outer layer is probably entirely absent from *Pecten*. Alternatively the fusion layer is not present and the epithelium secreting the outer layer is much more widespread than in other lamellibranchs, e.g. Tellina (Trueman, 1949, fig. 8), extending right along the epithelial ridges in the dorsal midline of the mantle (fig. 5). From a functional aspect this layer is directly comparable with the outer layer of other lamellibranchs. It is convenient to refer to it as the outer layer of the ligament of *Pecten* without prejudicing the possibility of this layer being the fusion layer. A detailed study of the mantle of *Pecten* in early post-larval life is required to elucidate this further. The possibility that the central part of the inner layer represents a much modified outer layer should not be entirely overlooked. A similar view was held by Newell (1937). It may be suggested that in such circumstances the mantle isthmus would be divided along the mid-line into two parts each secreting the lateral regions of the inner layer. The central part of the inner layer is, however, markedly different in appearance and structure from the outer layer of the ligament of other lamellibranchs and it appears improbable that this view is correct.

Apart from the structure of the outer layer discussed above, the ligament of *P. maximus* shows two important differences from that of other lamellibranchs; first the elongated outer layer and secondly the unusual non-calcified region of the inner layer. The significance of these may be related to their mechanical properties.

The outer layer of all ligaments is normally subjected to tensile stress when the valves are closed (the inner layer simultaneously to compression) and the removal of small parts considerably reduces the opening moment of the ligament. Experiments have shown that removal of such parts of the outer layer of *Pecten* does not always have a very marked effect (Trueman, 1953). Removal of all of the outer layer either from in front of or from behind the umbo causes the tearing of the inner layer and leads to the misalignment of the valves. Thus the elongate outer layer of *Pecten* is not primarily concerned with the opening strength of the ligament, but may be considered to behave as a fairly rigid hinge structure, related to the prevention of the rotation of the valves one about the other.

The ligaments of *Pecten* and *Chlamys* are particularly efficient for the rapid opening and closing of the valves, having a lower work-loss than those of other lamellibranchs (Trueman, 1953). This may be attributed to the non-calcified inner layer of the ligament. The calcified inner layer of *Lutraria lutraria*, for example, has a modulus of elasticity in compression approximately seven times the similar but non-calcified inner layer of *P. maximus*. Calcification increases the resistance of the inner layer of the ligament to compression, but it has been further observed that this is associated with an increased internal resistance.

The central non-calcified region is thus important in respect of the relatively high efficiency of the ligament. Such a structure is particularly well adapted to the rapid and frequent opening of the valves found in *P. maximus* and *C. opercularis* in association with the swimming habit.

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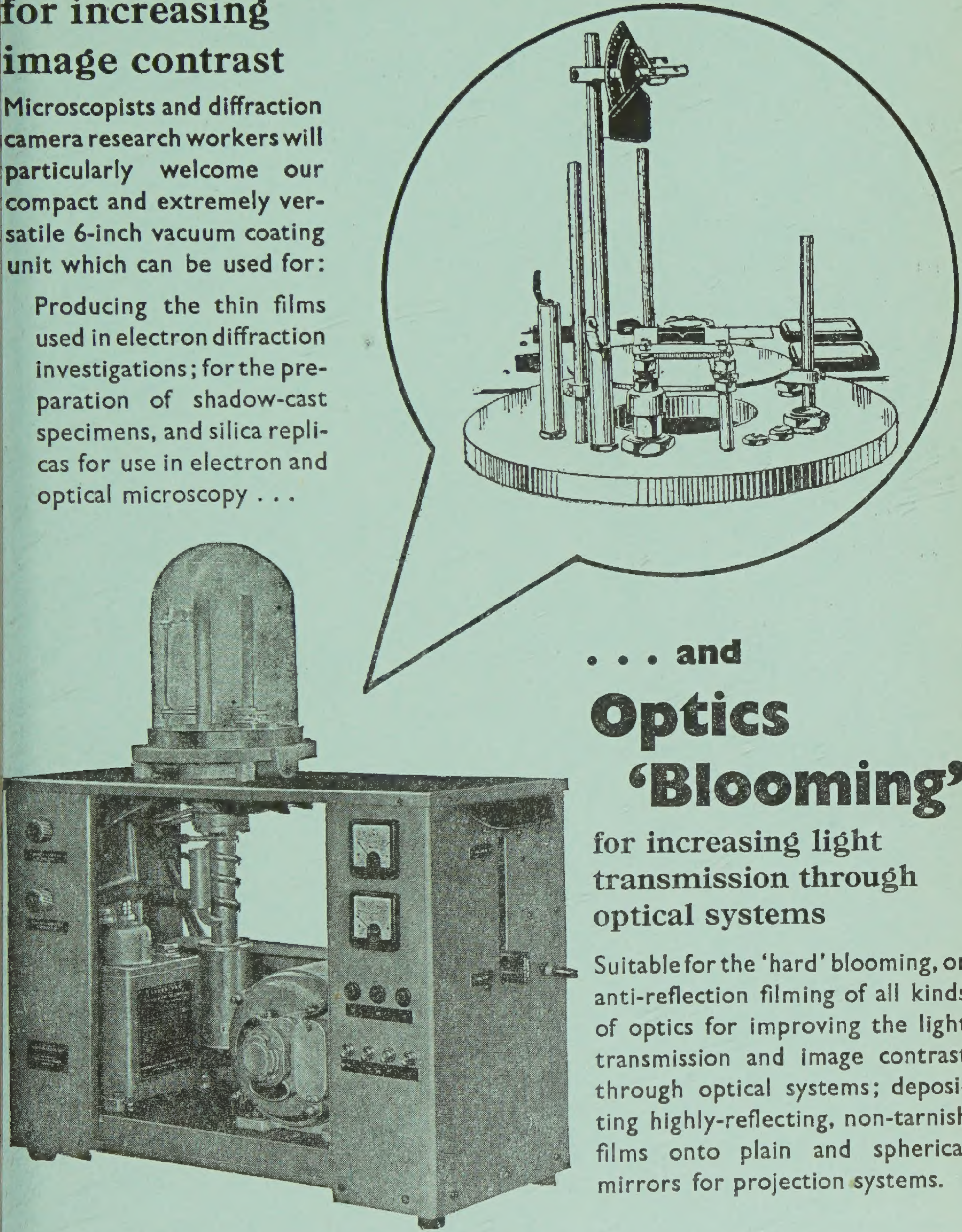

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